

Unreal market for petroleum

OPEC's meeting earlier this week will not have restored to the oil producers their lost dominance. Nothing can do that. But Britain and the United States should impart realism to their oil policies.

THE meeting earlier this week at Geneva of the Organization of Petroleum Exporting Countries (OPEC) is an important landmark in the industrial history of the recent past. As at other recent OPEC meetings, representatives of the thirteen member states will have been forced to admit that they are no longer in a position to achieve their common purpose, that of keeping the price of crude oil at something like what they were able to command in 1979, when the price escalation enforced in 1973 was reinforced, carrying the price of crude oil to \$32.50 a barrel (at 1979 prices). Since then, OPEC's prices have been constantly under pressure, and have indeed now drifted downwards, against the direction of inflation, to a nominal price of about \$28.00 a barrel, which is itself heavily discounted on the spot market for crude oil. This week's meeting was called to grapple with a problem now thoroughly familiar to OPEC members, that of striking a balance between the legitimate long-term interests of all OPEC members to keep the price of oil as high as possible and the more immediate interest of some members (such as Iran and Nigeria) that short-term revenues from the sale of oil should be maximized, which can be done only by selling oil below the official OPEC price. The outcome is likely to have been an awkward compromise, a fudge.

OPEC is not a cartel in the strict sense. The simplest proof is that the organization cannot enforce the compliance of its members with its central pricing policy, nor with its agreements on production quotas for individual member states. The old-fashioned industrial cartels of the 1930s were much more powerful, able to penalize those who broke the agreed rules. OPEC could never have acquired such internal discipline. Its members are, in effect, sovereign states which cannot indefinitely be bound by agreements they arrive at voluntarily. (Many of the troubles of the European Communities come about because only the provisions of the Treaty of Rome, and the regulations derived therefrom, can be legally enforced.) So the traditional cash-hungry members will go on shading whatever prices have been agreed this week. Even the stoics among OPEC's membership, such as Saudi Arabia, have now become sufficiently dependent on the income stream from oil that they will not easily be able to turn off the tap to benefit their hungrier fellow-members.

Weakness

OPEC's more serious weakness is that its joint production no longer dominates the international market for crude oil. Production from Mexico and the United Kingdom has increased during OPEC's ineffectual self-restraint while, over the past decade, domestic production in the United States has for practical purposes removed from the international markets what seemed in 1973 to be a new and clamant customer for crude oil. While the price amounted to only a dollar or so a barrel, there were clear advantages for the United States in buying from the Middle East, but at \$30.00 a barrel, Texan oil, even that from marginal wells, is much cheaper. During the same decade, other customers for OPEC oil have become much less eager buyers. Throughout the industrialized world, in Western Europe and Japan, the consequence of higher oil prices has been the predictable encouragement of fuel efficiency, and the encouragement of the use of other fuels. Especially because the strength of the dollar

(in which oil prices are denominated) has not yet allowed consumers outside the United States to sense that oil is becoming cheaper, the pressures in favour of conservation and substitution will be reinforced. So OPEC is certain to remain a toothless tiger for at least the remainder of this decade or at least until it reasserts its market power by undercutting independent suppliers of the international market, Britain and Mexico in particular.

What lessons can be learned from this sequence of developments? A long time ago, in the late 1950s, the price of crude oil from the Middle East was about \$1.50 a barrel, the revenues of the oil-producing governments were between \$0.10 and \$0.50 a barrel and the cost of crude petroleum was so low that, in both Japan and Western Europe, oil became the cheapest way of generating electricity. Towards the end of 1958, a committee appointed by OECC (now the Organization for Economic Cooperation and Development) under the late Sir Harold Hartley uttered the firm opinion that petroleum was so cheap, and the supply so apparently unclouded by uncertainty, that plans for the rapid development of nuclear power were unnecessary. Although OPEC came into being the following year, a further decade went by before it began to flex its muscles (with the nationalization of Libyan oil wells in 1969). Throughout this period, the oil consumers of the West were fecklessly indifferent to the notion that the oil producers were well placed to capture for their product a price not very different from that being paid for other fuels. The sequence of price increases decreed by OPEC in 1973 was an illustration of how chickens will come home to roost. Finding no resistance in the market to one price increase, OPEC simply went ahead and made another. Its error, then, was not so much that it sought in 1973 (and, later, in 1979) to make as much profit as possible for the oil it was able to supply, but to fail to recognize from the outset that the arbitrarily chosen price of oil must itself be as unstable as the artificially low price of the 1950s. The only price that matters is that at which willing buyers will buy from willing sellers. To judge from the noises at the opening of this week's meeting, OPEC has still not learned that lesson.

Substitution

With two exceptions, industrialized countries seem to have learned where their interests lie. In Japan and mainland Western Europe, the blend of conservation and substitution has made most countries less dependent on oil and less vulnerable to sudden changes of price. One striking feature of the past decade has been the growth of an international market in coal, while the supply of natural gas from the Soviet Union has also helped to take the edge off anxiety about the future. The exceptions are countries now stranded at the opposite ends of the economic spectrum, the United States and the United Kingdom. By the legal discouragement of both imports and exports of crude oil, the United States has managed to insulate itself from the international market, allowing domestic users to buy oil at prices less than those obtaining elsewhere in the industrialized world, which are effectively determined by the cost of winning oil in the United States (which is less than that in the North Sea although greater than that in the Middle East). In fact, it would make sense that the United States should allow itself to export crude oil in



whatever quantities it could sell at the going price; the result would be to reinforce the already impressive tendency towards fuel efficiency the past decade has seen, to reinforce the sense of realism that must dawn on OPEC and at the same time to take at least the edge off the horrendous deficit on the trade balance of the United States.

The British case is different. North Sea oil was merely a mirage when OPEC's existence began, but Britain has now become more than self-sufficient. But the circumstances of the United States do not apply. The cost of winning oil from the North Sea is much higher than in Texas or the Middle East, and although there is no reason why oil companies which have already invested in holes in this inhospitable ground should not sell the oil that comes up for what it will make, falling prices are bound to undercut the incentive for drilling further holes. That is the most durable reason why the past few months of falling oil prices have made the financial markets jittery about the health of the British economy but there is also a more immediate worry, the knowledge that the British government's revenues depend significantly, if only at the margin, on the tax levied on oil production in the North Sea. In the short run, as sterling falls against the dollar, the taxes (calculated in sterling) will not be vulnerable but, further ahead, the prospect that Britain, an oil producer for the past fifteen years, will soon cease to be a potential member of OPEC is bound to scare the international community. One measure of that anxiety is the further increase of 2 per cent in bank lending rates earlier this week — the second such step in ten days. Another is the general sense of unease that five years of a generally rigorous economic strategy have yielded nothing. There must be many in Britain now who wish that the North Sea had proved empty of oil, and that traditional British manufacturing industry had not been decimated by the uncompetitiveness engendered by the brief spell when British oil flowed freely. □

Spreading thin

The British research enterprise wants to spread £4 million fairly, which is impossible.

THE Royal Society, probably the most reputable means of pulling chestnuts out of fires, has now lent three of its officers (including its president, Sir Andrew Huxley) to what may yet prove a risky cause, that of helping the British University Grants Committee spend a windfall £4 million on university equipment. The windfall came about at the end of last year, when Sir Keith Joseph, Secretary of State for Education and Science, promised an extra £10 million for the provision of equipment at universities only to find that, before he could deliver, more than half the money had been snatched away again by the government to which he belongs, on the grounds that the source of the promised funds (the pockets of middle-class parents) was politically a poisoned chalice. So the grants committee is faced with the prospect of distributing a sum of money so small that a single good use could absorb the lot among as many recipients as possible, so as to encourage the notion that fair play is not yet dead. To assist the president of the Royal Society and the physical and biological secretaries thereof, not to mention the chairman of the grants committee and the executive head of the Medical Research Council, there will be seven other distinguished academics. Their task is to pick a dozen research groups in the United Kingdom to which the committee may make a substantial present of equipment.

The obvious danger in the arrangements now in place for distributing what is, after all, a very modest sum of money, is that even this distinguished committee will overlook some worthy cause, a group of talented researchers somewhere whose claims on shrinking public funds are by accident neglected. The contrary risk, that the committee will somehow seize on projects to support whose virtues are more apparent than real, is not serious but cannot be neglected. That is one potential source of risk. It is, however, much more a source of hazard to the public reputation of science and those who manage it in Britain that such a large

group of such distinguished people should be engaged in a once-and-for-all attempt to apportion £4 million among what must be 1,000 legitimate claimants. Especially because the grants committee has set up three separate committees to decide how, and by what criteria, particular university departments should be encouraged over the long run, there is every reason why the members of the extra committee now appointed should have declined to serve, giving the excuse that they had more important things to do.

Unfortunately, this is not how the administration of British science works. When funds are as scarce as they have become, so much so that groups of talented people are at their wits' end to know how they will prosecute their ideas, there is no way in which an extra £4 million can put things right. Yet everybody behaves as if fairness must be demonstrated. So, small though the occasion may be, the committee must be all the more distinguished. Drawing lots would serve as well. □

Congress and science

Congressmen should know some science. US universities are cashing in.

UNIVERSITY public relations, also known as the art of reflecting glory, took a major step forward several years ago when Harvard University's Kennedy School of Government hit upon the scheme of staging a special seminar on public policy and legislative procedure for newly-elected congressmen. Other universities have since seized upon the concept, recognizing their unique ability to provide such a public service as well as their unique ability, unshared by the National Association of Dredging Contractors, to lobby under the guise of educating.

The latest entry is George Washington University, which last week put on its first Seminar on Science in Government for new members of Congress, a two-day cramming session organized by Mike McCormack, himself a former congressman and now a Washington consultant on science policy. Leading lights of science policy and science-in-society, among them Joshua Lederberg, Lewis Thomas and Leon Lederman, were poised at hourly intervals to brief the dozen or so congressmen who showed up.

First, however, the president of George Washington University reminded the congressmen that if they ever needed technical information, they need only pick up the phone and call George Washington University. The National Academy of Sciences (NAS) representatives (NAS supplied the meeting rooms for the seminar and the accompanying social events) reminded the congressmen that the briefing documents on interesting areas of science prepared by the academy for science adviser George Keyworth were available to them. The representative of the American Society of Mechanical Engineers reminded the congressmen of his group's sponsorship (and origination) of the Congressional Fellows Program, which provides expert mid-career scientists and engineers free of charge as congressional staffers for one year. Mike McCormack reminded the congressmen of the sponsors that made the seminar possible, among them Bechtel, Atlantic Richfield, Dow Chemical, Monsanto, Stone & Webster and the US Committee for Energy Awareness.

The congressmen learned the first day that "science does not involve partisan politics"; that "science is not a panacea"; that two-thirds of the world's population lives in poverty; that the build-up of carbon dioxide in the atmosphere and nuclear war threaten mankind's survival; that "there has never been a scientific problem more urgently in need of settling" than that of nuclear winter; that "scientists come in two classes, good ones and not so good ones"; that people with PhDs can be "dumb"; that "predictions can often be wrong, particularly if they're about the future"; that one senator in 1901 complained that he was tired of "all this science" on which millions had already been spent and it was "about time it was stopped"; that the first law of thermodynamics can be derived from symmetry considerations; and that instead of multiplying two factors you can add their logarithms. By then there were only four congressmen left. □

NIH research

Administration makes rings around congressional edict

Washington

THE Reagan administration's Office of Management and Budget (OMB) has hatched what is being termed a "creative" scheme for undoing Congress's decision to increase the number of new research grants awarded by the National Institutes of Health (NIH).

Last year, Congress repeated its usual pattern of going one better than the administration in generosity toward NIH. It added some \$580 million to the Reagan request for \$4,400 million, already an all-time high. Along with the congressional pet projects (\$5 million for five new Alzheimer's disease centres, \$10 million for small universities and the historically black colleges, \$34 million for new clinical trials) there was an extra \$280 million for research project grants. The appropriations committees in both houses of Congress agreed that this money was for two purposes: increasing the size of each grant to the "historical level", and increasing the number of new and competing research grants each year to 6,500.

The present 5,000-grant level, long an informal target for NIH, has more recently been effectively a ceiling. Congress was persuaded to increase the number of grants by testimony showing that many projects approved by study sections were neverthe-

new grants to 6,500 per year would automatically require further budget jumps of some \$300 million for each of the next two years as well. The challenge for OMB was thus to devise some way of disposing of the money Congress had already appropriated for the current fiscal year that would not set a precedent for future increases.

The solution, undisguisedly contrived, may be difficult for Congress to challenge; OMB has ordered NIH to award only 5,000 new grants this year and has arranged that the extra cash will be set aside at once to cover the full three years of as many of those 5,000 grants as it takes to use up all the money (about 700 grants). Thus 4,300 grants have been appropriated funds for one year, according to OMB's reckoning, and 700 for all three years at once.

Congress usually appropriates only one year at a time; congressional staff members have already questioned the legality of OMB's action. But OMB may get its way. Congress's directive to increase the number

of grants is not written into law, but appears only in the committee report. Such report language is considered indicative of congressional intent; legally, however, it is only part of the "legislative history" — the body of clarification that courts may look to in determining the intent of Congress. Congress has indeed taken such matters to the courts on occasion, but in this case, by the time the case could be heard, the fiscal year would probably be long gone.

OMB officials have revealed that the fiscal year 1986 budget request, to be released by the President this month, will also call for holding the line at 5,000 new grants. If Congress wants to be sure to have its generous way this time, it will probably have to write its intentions into law. But that is easier said than done: a multi-year effort to write new authorizing legislation for NIH ended in a presidential veto last fall because of administration and NIH opposition to its provisions, spelling out in detail the research missions of the various institutes and creating two new institutes that NIH did not want — one for arthritis and one for nursing. And putting the 6,500-grant requirement into law would mean reopening all those issues, as well as some perennial Senate favourites, such as limitations on NIH-sponsored fetal research.

Stephen Budiansky

Space telescope

Users agree on inside track

Washington

ASTRONOMERS with guaranteed observing time on the Hubble Space Telescope, as the Large Space Telescope is now called, met in Princeton last week to stake out their positions and concluded cooperative agreements dealing with everything from data sharing to press relations. The meeting, convened by John Bahcall, did not become the "bloodbath" some had feared, but, by eliminating duplication, increased observing opportunities. At a meeting the previous week in Tucson, astronomers had been divided over whether time allocated to guest observers should include large slots for "key projects".

Each of the seven guaranteed observer teams (one each for the six instruments plus one "observatory" team) has been assigned 400 hours of observing time by the National Aeronautics and Space Administration (NASA). Until the Princeton meeting, teams had jealously kept their plans secret from one another, but have now decided to "behave like gentlemen", according to Bahcall.

One problem is that immediate public recognition is likely for the first space telescope observations of such interesting objects as galaxy M-87, whether the hypothesized black hole is detected at its centre or not. In order to avoid rivalries, written agreements were signed to share press opportunities and to delay publication in academic journals until colleagues have

seen results. Guaranteed observers' proposals, which will now be substantially reworked, will be formally submitted to NASA on 31 March.

In Tucson, six working groups reported on their deliberations on the desirability of key projects for up to 30 per cent of guest observer time. Although everyone accepts that some programmes will last longer than others, there were "spirited discussions" on how they should be designated and what they should be. While it seems that six key projects are now being considered, there is concern that the process should not prejudice new ideas. A meeting for potential European guest observers is to be held shortly in Munich; in the meantime, no final decisions on key projects have been reached, according to George Field of Harvard University, chairman of the space telescope advisory committee.

The telescope, which has been subject to several delays and cost overruns, is now being assembled by the Lockheed Corporation in Sunnyvale, California. Although it is scheduled for launch on the shuttle in August 1986, less optimistic observers, such as Richard Harms of Applied Research Corporation, who developed the faint object spectrograph on the telescope, doubt that the instrument will be ready in time. No outstanding technical problems are recognized, but, historically, problems do tend to surface during final assembly.

Tim Beardsley



less not supported. The new money became available to NIH at the start of the present fiscal year, 1 October 1984.

The administration, however, wanted to keep the number of grants at 5,000 and OMB is apparently not taking no for an answer. In its hunt for cost-cutting measures to reduce the \$200,000 million annual federal deficit, OMB has singled out the projected growth of NIH grants. Because grants are awarded for three years, Congress's decision to raise the number of

Heart transplants

Japan tries limited experiment

Tokyo

JAPAN's Ministry of Health and Welfare (MHW) is to back a major new project aimed at resuming heart transplants in Japan, almost 17 years after the nation's first heart transplant caused a public outcry and brought further attempts to a halt.

In August 1968, Professor Juro Wada, then at Sapporo Medical College in the northern island of Hokkaido, performed the nation's first heart transplant. Overnight, he gained much the same fame as Christian Barnard had in the west. But things quickly went sour. Although the heart recipient lived for 82 days, which was a considerable period of time given the state of knowledge in 1968, the press turned on Professor Wada — actually even before the recipient's death — and subjected him to savage personal criticism. A complaint was filed against him that the donor had not been dead at the time the heart was taken — the legal criteria of brain death having not been clearly established — and that he was thereby guilty of murder; but no prosecution was ever actually carried out.

What exactly lay behind this campaign of vilification is a complicated tale; regrettably, it was in part encouraged by doctors at more famous medical schools who had wished to claim the honour of the first transplant for themselves.

But something rather more illogical than envy was also important. There remains in Japan a peculiar and inexplicable opposition to organ transplants, to the extent that it is extraordinarily difficult to find heart, kidney or eye donors. Quite why this is so, nobody knows. Some say it stems from Buddhist beliefs about the interment of bodily remains: after all, each year there are several expeditions to sites of fierce Second World War battles, such as Saipan and Iwo Jima, to search for the bones of long-dead Japanese soldiers so that they can be brought back to the homeland. That bodily remains matter so much does seem to be rather cast into doubt by reports from the Railway Lost Property Office, however — each year around 15 Buddhist funeral urns are left on trains and never claimed.

Others seeking to explain opposition to organ donation point to a Confucian influence with emphasis on the notion that having been given one's body as a precious gift from one's parents, one is not free to give parts of it away. The absence of the Christian idea of charity may also be to blame; donating things to people one does not know or has no connection with is a custom only just beginning to take root in Japan. Also there is perhaps a tradition that sudden death is preferable to a long battle in a hospital. Again, the government has never made a serious attempt to introduce a "donor card" system. Most probably all these influences converge. But what adds a twist of further illogicality to

the Japanese situation is that very recently a Japanese went to the United States and successfully received a heart transplant from a US donor. Press reaction was ecstatic, despite the fact that the very same newspapers criticize attempts to restart heart transplants in Japan.

One result of the unwillingness of the Japanese to donate organs has been a major effort to develop artificial equivalents. Japan now has the highest number of kidney machines per head in the world and relies upon them in 98 per cent of cases of kidney failure. In other advanced countries, transplants are carried out in 50–75 per cent of cases: without its own supply of kidneys, Japan has instead to rely on machines and US imports obtained at weekends and public holidays when few kidney transplants are carried out in the United States.

Artificial heart research is also very advanced. The group at the Tokyo University Institute of Medical Electronics, headed by Professor Kazuhiko Atsumi, is a world leader with a record-breaking goat that stayed alive for 344 days with a totally artificial heart. Groups at Osaka, Kyoto and Fukushima follow closely behind. Artificial hearts are at present driven by an external source of compressed air but the Tokyo team believes a totally implantable compact heart with its own power supply could be ready for animal testing in 3–4 years and a finished product in ten years.

Final development of an artificial heart will occur, though, only if heart transplants also become common in Japan, for their first use is likely to be to keep a patient alive until a suitable donor heart can be found. The new MHW projects will try to win consensus for resumption of transplants; indeed, it is already rumoured that several transplants will be performed this year. A hundred million yen has been promised by the ministry: first goals will be to collate information on heart transplants throughout the world to help win over public opinion. Opinion has already been sounded in the medical profession and 90 per cent of doctors in medical schools are now willing to accept "brain death" as a criterion of death. Two hundred doctors have joined a society for heart transplants and 300 that for artificial hearts, both established very recently. Research will then be promoted on transplants in animals, organ preservation, a rapid distribution system for donated hearts, immunological control mechanisms and artificial hearts needed to back up operations.

Even if a national consensus can be won, it is still unlikely that Japan will ever be able to supply all the organs it needs. Unlike the United States, there are just not enough young people dying violent deaths or being killed in traffic accidents to supply the organs required.

Alun Anderson

Hannover Fair

Endless shop-window

THIS year's Hannover Fair, to be held in West Germany from 17 to 24 April, will be the biggest ever, with more than 6,700 exhibitors from 46 countries. Last year, 740,000 visitors attended in the eight days of the fair and considerably more are expected this year. The demand for exhibition space has now so far outstripped the provisions of the site that from 1986, the office and data technology fair will be held separately, three weeks earlier, to allow other parts of the fair further room for expansion.

The largest trade and industry fair in Europe, Hannover is known as the Fair of Fairs, because it is made up of ten separate trade fairs run concurrently. By bringing together so many aspects of industry, ranging from microelectronics and robotics through electrical and mechanical engineering to cleaning technology and waste disposal, a great opportunity for cross-fertilization is provided. Nowhere is this more apparent than in the research and technology exhibition, unique to Hannover and one of its most popular attractions. Behind this innovation, started in 1976, is the concept of horizontal technology transfer — that many solutions to specific problems can have much wider applications than their inventors ever conceive of. A good example is the inventor of the air pump, who surely never foresaw that it would become an essential part of the compressor in millions of refrigerators. The exhibitors displaying such developments can attract an enormous range of potential customers, who in their turn have the chance to discover solutions to technical problems that have been impeding their development of new products.

This year, for the first time, foreign universities will be exhibiting, in addition to 46 German universities, technical colleges and research institutes. Here, demand for space has now outstripped provision by 30 per cent, and next year the research and technology exhibition will be housed in a far larger hall. The concept of "researcher" also has to be seen in a wider context than those working in laboratories and research institutes, as many individuals now exhibit their specialized technical inventions at Hannover, hoping to find a manufacturer interested in large-scale production.

A further development of the technology transfer concept is the new biotechnology exhibition, BioTechnica, to be staged for the first time on 8–10 October 1985. Eighty or more exhibitors from Europe, the United States and Japan will show marketable biotechnological research findings, new products and methods as well as laboratory and production techniques necessary for the practical application of their developments.

Jennifer Altman

Pacific oceanography

Franco-Japanese plan mounted

THE French deep-sea submersible, *Nautille*, designed to reach a depth of 6,000 metres with three scientists aboard, has behaved "beautifully" in its first trials in the Mediterranean, according to its test-pilots. Thus all augurs well so far for *Nautille*'s real business this summer, the intended investigation of the deep-sea trenches off Japan, where in the south the Philippine plate, and in the north the Pacific plate, plunge under the continental Eurasian plate on which Japan is sitting.

According to the French co-director of the project, Xavier le Pichon of the University of Paris VII, *Nautille* will first carry out a seven-day trial run in the Puerto Rico trench at 6,000 metres, but there will be little time there for research. But in Japan in June and July, *Nautille* should make possible some unique geological observations on one of the most active subduction regions in the world. And quite apart from the geotectonics, which are believed to be threatening a magnitude-8 earthquake close to Tokyo in the near future, *Nautille* will be searching for subduction-zone analogues of the "vent communities" of sub-ocean animals discovered by the US submersible *Alvin* at spreading centres in the Pacific.

There are already indications, in the form of observations of up-welling mud from Japanese trenches, that there may be vents in the subduction zones, le Pichon says. Evidence was presented at the American Geophysical Union meeting in December of dense communities of tube worms and clams recovered by *Alvin* along the Oregon-Washington subduction complex. Bottom waters there, at a depth of 2,036 metres, are rich in methane, Dr Erwin Suess of Oregon State University reported, and the chemistry suggests processes involving methane oxidation.

Thus the phenomena that *Nautille* will be looking for are quite different from those in spreading centres, where hot sulphur-rich waters drive anoxic benthic communities. In the case of subduction zones, the processes seem likely to involve the extrusion of relatively cool pore waters from sediments rich in organic debris.

Geologically, one of the most important tasks of the Kaiko (Japanese for "trench") Franco-Japanese collaboration with *Nautille* will be to place a tilt-meter and seismometer, both Japanese-designed, at a depth of 2,500-3,000 metres near the Bonin ridge. This volcanic arc runs south-west of Tokyo, and ends on the landward side in the Izu peninsula — which is resisting subduction under Japan. It is a release in the stresses in this zone that most threatens the Tokyo region with major earthquakes, and the instruments to be placed by *Nautille* are designed to help predict movements (with the national earthquake prediction network, see *Nature* 312, 500; 1984).

But M. le Pichon describes the work to be done in placing these instruments as somewhat difficult — involving using *Nautille* to drill post-holes in the rock — and an oceanographic "first", so nobody is yet entirely certain that the exercise will be successful.

Other work to be done by *Nautille* includes the direct observation of the subduction of two seamounts, one of them as big as Mt Fuji and already half-digested by the Japan trench, and a study of sediment deformation in the highly-sedimented

Nankai trough, where the Philippine plate meets the Eurasian plate.

Planning for the expedition began a decade ago, according to M. le Pichon. Operating costs for the two months' observation (some £2 million) will be divided equally between France and Japan. The construction costs of *Nautille*, borne by the French oceanographic agency the Institut Français de Recherche Pour l'Exploitation de la Mer (IFREMER, formerly CNEO), are estimated at "tens of millions" of pounds. With a diving capability of 6,000 metres, the vessel should be able to reach some 97 per cent of the oceans' deepest waters, it is claimed.

Robert Walgate

NASA

Perpetual plane in prospect

Washington

A PERPETUAL aircraft is being planned in the United States. The National Aeronautics and Space Administration (NASA) will shortly award \$375,000 for a feasibility study of a novel high-altitude atmospheric sensing vehicle, called the Carbon Dioxide Observation Platform System (CO-OPS). The novelty is that the unmanned aircraft would be given very long endurance by means of microwave power beamed up from ground stations.

As the project is still very much up in the air, nobody wants to appear too closely committed to it. Fred Koomanoff of the Department of Energy's carbon dioxide research programme says the idea for CO-OPS was volunteered by Charles Guttman at NASA's Marshall Space Flight Center in Alabama. Guttman, however, when asked about the CO-OPS concept, said "there is no concept". NASA is simply following up on a Department of Energy study on the use of space for research on carbon dioxide in the atmosphere.

Koomanoff speculates that a long-en-

durance craft flying at 60,000-70,000 feet could be the best way of obtaining some atmospheric data; unlike balloons or manned aircraft, it could stay in nearly the same place for long periods. CO-OPS might measure atmospheric aerosols, concentration of carbon dioxide and trace gases in the atmosphere and obtain temperature and pressure profiles. CO-OPS could also observe land and sea ice and measure albedo. Military use is possible.

Some at NASA are sceptical about the use of microwave power, however, on the grounds that safety considerations could be limiting. Nevertheless, NASA's bid for proposals virtually rules out solar power and chemical energy for CO-OPS because of weight considerations, while low-cost phased-array transmitter technology "appears feasible".

Potential contractors are reminded that CO-OPS would be a low-cost project using proven technology, to fly in perhaps 5 or 6 years' time. The closing date for proposals was 30 November 1984.

Tim Beardsley

Ocean Drilling Project setback

BRITAIN has withdrawn from the Ocean Drilling Project (ODP), because money for the annual subscription of £2.5 million could not be found in time for the January deadline. The only hope now is that, with appropriate juggling of accounts and perhaps some arm twisting, the United Kingdom could rejoin from next October.

Canada, France and West Germany have already joined ODP and Japan has said that it will join from next October; the project is run by the Joint Oceanographic Institutions and is administered at Texas A & M University. ODP is the successor to the Deep Sea Drilling Project and involves a new ship known variously as "JOIDES Resolution" or the Sedco/BP 471, now completing its "shakedown" voyage.

For more than a year, the UK Natural Environment Research Council (NERC),

together with the Department of Education and Science, has been trying to persuade the Department of Energy and members of the oil industry to top up NERC's contribution to reach the full subscription. A spokesman for NERC acknowledged that they had been successful in raising funds but that an unspecified gap still remains. It is hoped that, by deferring payment of the subscription until October, NERC can meet ODP's financial requirements. In principle, all British scientists should now come off the ODP committees but it is hoped that, if NERC can juggle its accounts (which would probably require Treasury approval — a commodity notoriously hard to acquire), a letter of intent could be sent that would permit some level of UK involvement in the interim.

Philip Campbell

US research contracts

Small may mean nuisance

Washington

MANY research institutions in the United States are expressing concern at a recent patent law requiring them to give preference to small businesses when licensing inventions arising from federally-supported research. One complaint is that many research universities are ill-equipped to evaluate the commercial strength of a small company eager to develop a potentially lucrative invention. And, according to draft regulations, would-be licensees who believe they have been treated unfairly can call for an investigation by the US Department of Commerce, raising the fear of harassment in research institutions.

The requirement that federal research contractors (other than large companies) must give small businesses first choice of patent-worthy inventions is encoded in public law 98-620, signed by the President in November last year, which gave the Department of Commerce responsibility

for administering the law and for producing regulations. The draft regulations now being circulated are nevertheless softer than some had feared: contractors are required only to use "efforts that are reasonable under the circumstances" to attract small business licensees, although the requirement to prefer a qualifying small business applicant remains. In practice, this means that in cases where no small business is likely to be able to take on development, for example of a potential new drug, the search need be only a formality.

Norman Latker, director of the federal technology management division at the commerce department, says the department "has not placed all that much emphasis" on the need to seek small businesses, and the law has been "viewed as a statement of wish". The department has stopped short of placing on research contractors a requirement to demonstrate that reasonable efforts have been made.

At one stage, it was feared that an unfavourable ruling following a complaint of unfair practice to the commerce department would mean that contracts with large companies would have to be cancelled. It is now clear, however, that the department will investigate only general licensing procedures and will not intervene in specific cases.

The public law 98-620 changed markedly during its passage through Congress. It was originally an attempt to extend to large companies the rights previously enjoyed by small businesses and universities to take out patents on invention arising from federal grants and contracts. In the event, there was political opposition in Congress and large companies now negotiate separately over patent rights with the federal agencies. But the provisions of the bill originally intended to give small businesses crumbs from bigger tables, applying also to universities, have survived.

Some provisions of 98-620 are, however, viewed as liberating. Major institutions owned by the government but operated by contractors, such as the Stanford Linear Accelerator Center and the Jet Propulsion will henceforth have the same first rights to patents arising from their research as independent contractors. Institutions in this category, which are owned mainly by the Department of Energy, also include Los Alamos, Lawrence Livermore and Argonne National Laboratories. And in another change, exclusive exploitation agreements with large companies will in future be permitted to extend up to the full 17-year lifetime of the patent. There had previously been an upper limit of five years from commercial availability of the product or 8 years from the date of the licensing agreement, a restriction that came to be viewed as an impediment.

Tim Beardsley

British research councils

Corporate plans all the rage

THE British Natural Environment Research Council (NERC) has come in for criticism over its handling of its own corporate plan — its proposals for structuring the next five years of its activities. The leaking of details of a draft version has resulted in a flood of letters to council members from interested parties. The outcome of a council meeting last Thursday is an unscheduled meeting called for this week which is expected to ratify a final draft for public release on 14 February.

There are three substantive issues involved in the proposed reorganization: centralization of control of the research institutes, the scale of staff cuts and the transfer of funds to support research in universities. NERC is adamant that the principles of the changes are already firmly established (and indeed were approved by the council last year), but that detailed modifications can still be incorporated in the light of opinion. The research council sees the corporate plan as a framework for future developments that will evolve during this coming financial year. There is still dissent amongst those who have discussed the document over the methods to be used to achieve the reorganization, but all parties do agree that fundamental change is necessary.

The problem is exemplified by the position of the British Geological Survey (BGS) as described by Professor J. Sutton of Imperial College in a recent speech critical of NERC. BGS is the only national survey under the aegis of a research council and stands in odd contrast with the Ordnance Survey (concerned with topographical maps), which comes directly under the Ministry of Defence. Elsewhere, in the United States, for instance, both topographical and geological mapping are functions of the geological survey of the Department of the Interior. As Sutton said, the comparable size of BGS and its parent body NERC causes problems with the result that senior members of BGS feel that independence is a viable alternative to the centralized direction now proposed.

One council member favourable to the plan said this week that geology has changed, with many important functions such as seismic surveying and mapping of the ocean floor requiring cooperation between BGS and, for example, the Institute of Oceanographic Sciences. Both are now components of NERC, and there is support on the council for this to continue, whatever reorganization takes place. Many prominent members of the British earth sciences community are calling for a return of BGS to its "proper" role as a national survey, providing and revising geological maps and a synthesis of the British geology.

Peter Gambles

New US-Soviet links

Washington

THE National Academy of Sciences delegation to Moscow last week reached a quick agreement with the Soviet Academy on a two-year cooperative agreement restoring many of the exchange programmes suspended by the National Academy in 1981 in protest against the internal exile of Dr Andrei Sakharov. A draft protocol signed by both delegations at the end of two days of meetings specifically calls for future cooperation to be in "forefront fields" of science in which both the United States and the Soviet Union are advanced. Those who will participate in these programmes, which are to include joint workshops and exchange visits, will have to be approved by both sides. These stipulations seem intended to answer past criticism that the Soviets avoid sharing the work of their best scientists, especially in fields such as mathematics where they may hold an edge over the United States.

The actual fields to be included under the new agreement have yet to be decided; each side proposed a dozen topics last week, but the US academy has declined to provide further details pending the completion of sensitive negotiations that are expected to lead to a final agreement within a few months.

A short statement released by Frank Press, the president of the National Academy, who led the delegation, made only a fleeting reference to the issue of Sakharov: "Our discussion also covered what constitutes a propitious climate for cooperation... This climate naturally includes our concern about human rights."

Stephen Budiansky

UK expenditure

Cash limits limit change

THE British government's spending plans for the next three financial years (the first, that beginning on 1 April), published last week, contain no surprises for research and higher education. The annual planning document (Cmnd 9428-1 and 2) confirms the details announced piecemeal in the past few months about the projected total of spending on research and higher education in the period immediately ahead. But even before the ink on the new document is dry, it seems to be generally agreed that the government will not be able to hold to its present course without further economies.

The document thus records the increase of public spending through the science budget agreed last December and amounting to an extra £11 million (compared with last year's estimate) in the year beginning 1 April, with increases of £8 million in each of the two succeeding years. In cash, this implies that the total science budget, that which is administered by the Advisory Board for the Research Councils, will increase from £550 million this year to £584 million next year and to £600 and £610 million in the two succeeding years when the rate of inflation is assumed to be 4 per cent and 3.25 per cent respectively.

Superficially, these figures imply that there will in fact be reductions of the budgets available for the research councils in the two years concerned. Apart from the assumptions made about the inflation rate, there is no assurance that the future pay settlements will tally with the government's assumptions.

On higher education, the latest figures suggest that the government is planning not for reductions of student numbers but for stagnation over the next three years. Student numbers at British universities and at other higher education institutions in England are expected to decline by 3,000 in the coming financial year but then to be effectively constant.

British government support for industrial research and development through the Department of Trade and Industry is also forecast to grow less quickly than inflation in the three years ahead. The government's white paper says that spending (estimated at £383 million during the current year, and covering the costs of the department's laboratories, existing grant schemes and support for space and aerospace) will grow to £390 million in the two succeeding years. The future of the British government's support by means of grants for industrial research and development is said to hang on the outcome of a review due to be completed before the end of March.

Defence research and development, some 12.5 per cent of the total defence budget (now running at £17,250 million a year), is not analysed in detail in the new survey of forward spending plans, but will be dealt with in the formal statement on the defence

budget due next month.

In their general character, the new budget plans contain no substantial departures from the British government's declared intentions on research. Of necessity,

British education

Lords against specialism

A STRONG attack on the British government's arrangements for technical education and training was launched earlier this week by the House of Lords Select Committee on Science and Technology. Among other things, a subcommittee under Lord Gregson argues that there should be new arrangements for enabling graduates to acquire the skills needed in the application of new technologies (*Education and training for new technologies*, HMSO, £6.00).

The general line followed by the subcommittee is that the essence of new technology is its unfamiliarity, for which reason the most appropriate preparation is a broad education. In this spirit, its complaints at the narrowness of the school curriculum in England and Wales. Both in the schools and in higher education, the devolution of responsibility to separate teaching institutions, acknowledged as a source of stability within the educational system, is also cited as one reason why valuable innovations do not spread quickly.

On the educational needs of the present, the committee argues for a more general curriculum at the early stages of people's development, saying that the needs of information technology can be met by education in mathematics, physics and English. Emphasizing the present shortage of trained people in information technology estimated by some of those giving evidence to amount to 5,000 graduates, the committee argues that there is an urgent need not merely for better provision for continuing education but for a better recognition of the need.

The central recommendation of the House of Lords committee is that there should be established within the framework of the Science and Engineering Research Council an organization to be called the Education and Training Board whose functions would include the financial support of vocationally oriented courses for graduates from British universities and other institutions. The board would also be responsible for surveys of manpower needs and for general advice on the design of courses.

More generally, the committee is critical of the *ad hoc* character of the British government's approach to technical education, pointing out that budgets for innovation are most likely to be cut first when funds are scarce. It recommends tax incentives

to encourage industrial and business support for education, but says nothing on the more contentious issue of whether people seeking technical education outside the limits of what the state provides should be able to offset the cost against taxation. In passing, the House of Lords committee supports the argument that British university research is in poor shape and urges that the pursuit of excellence in research necessary for the successful exploitation of new technology. □

Seminar scrapped

Los Angeles

THE chief surgeon at the School of Medicine at the University of California, San Diego, last week cancelled a seminar demonstrating new surgical techniques on dogs after receiving a death threat from an anonymous telephone caller. According to university officials, this is the first time any such event has been called off in a California state school because of complaints of animal abuse in scientific work.

The surgeon, Dr A.R. Moossa, had planned to teach a seminar this month showing physicians how to use staples to save operating time. Thirty-six anaesthetized dogs would have been put to death after the demonstration. On 14 January, a man telephoned Dr Moossa's office and, according to Sharon Gillespie, the surgeon's assistant who took the call, threatened to put a "bullet in (Moossa's) head" if he did not "stop the thing with the dogs". The caller did not identify himself nor did he name any animal rights group, the surgeon's assistant said.

Dr Moossa promptly cancelled the seminar. Although this is the first time he has been threatened with bodily harm, Dr Moossa has received half a dozen letters protesting against the use of animals in surgery. The dogs were to be obtained from the San Diego county animal shelter and would have been destroyed anyway, according to a university spokesman.

Many California researchers are beginning to feel threatened by the growing militancy of animal rights groups, according to Sandra Bressler, executive director of the California Biomedical Research Association, an organization set up in part by the state's universities to counter the animal rights protesters. Sandra Blakeslee

British fund-raising

Hard sell in cancer research

MAILING shots from a British cancer research charity called the Association for International Cancer Research (AICR) have been descending on surprised members of the British public. The letters begin by inviting recipients to take part in a prize draw, and say that luxury items worth up to £10,000 are being given away. The letters, carrying a return address at a Cheltenham post office box, are signed by Frank Salisbury, and go on to suggest that appreciative recipients may wish to make donations to AICR's fund for cancer research. Failure to do so, however will not jeopardize chances of winning a prize. A spokesman for the mailing house said this week that the full address was omitted by accident from the first mailing pieces.

The hard sell on behalf of a good cause is unfamiliar in Britain, although the technique of linking a prize draw with a solicitation has also recently been used by the Consumers Association on behalf of its publication *Which?*. Those familiar with British cancer research charities have also been surprised that AICR's account of recent progress in cancer research closely follows a document put out last year by the Cancer Research Campaign.

AICR began life in December 1979 as NFCR-UK, a British spin-off of a US organization, the National Foundation for Cancer Research (NFCR). The declared objectives of NFCR-UK were to "promote, conduct and support research into the causes, prevention, treatment and cure of cancer and associated conditions". The directors were Franklyn Salisbury, his wife Tamara, Professor Kenneth Rees of University College London and Professor Trevor Slater of the department of biochemistry at Brunel University.

At an extraordinary general meeting in December 1982, it was decided to change the name to AICR. The recent mail-shot seems to reflect AICR's wish to sustain itself through "our own fund-raising efforts in the UK". At the outset, NFCR-UK received £450,000 from its US parent organization. Since then, annual expenditure on grants has not exceeded accrued annual interest. The mail-shot describes recent developments in cancer research in a manner which, by elisions of syntax and layout, prompts the inference that AICR is responsible.

NFCR was set up ten years ago in the United States by Salisbury, an east coast lawyer, with the help of Dr Albert Szent-Gyorgyi, the Nobel laureate. Last year, Salisbury was granted an audience with the Pope in recognition of services to cancer research in Italy. Later this year, he will be awarded an honorary degree by the University of Wales. His present involvement with AICR may explain the aggressive style of its fund-raising campaign. NFCR has made grants to more than 80 laboratories in 15

countries. Its international status has complicated its task of complying with US regulations governing charities which, *inter alia*, require that each year's income should be spent or overspent in the year in which it is earned.

From its inception, AICR has been coordinated from Brunel University. Professor Robin Willson, whose research there into the interaction of certain drugs with oxygen-free radicals has been supported by NFCR, explains that a decision was made at the last annual general meeting to relocate AICR's administrative centre at the University of St Andrews, under the auspices of Dr Colin Thomson.

Dr Thomson says that the prize-draw invitations were sent out to 400,000 members of the public believed to be susceptible to mail-order solicitations. According to Dr Thomson, an additional leaflet outlining recent scientific advances was sent out to 10 per cent of those on the mailing list, but as an experiment.

When asked why AICR had embarked on this campaign after four and a half years of relative inactivity, he replied that "the previous board didn't think it would work here. The kind of reaction one is inevitably going to get from the established groups is that we're only interested in the kinky

fringes of science. In fact we aim to focus attention on novel and under-investigated areas of research."

Professor Willson, however, fears that AICR's new tactics may detract from the work of established groups. "Our only doubts about raising funds in Britain are that we do not wish to tread on the toes of the Institute of Cancer Research and the Cancer Research Campaign." Dr Thomson dismisses this concern; "we're trying to support good science, high-quality research which is not competitive but complementary".

Apart from research supported by the British fund, some groups continue to be supported directly from the United States. Dr Ronald Pethig of the University College of North Wales at Bangor says that the US fund has supported his investigation of cell membrane structure to the tune of £40,000 a year for several years generously and flexibly.

On the telephone from Washington this week, Mr Salisbury said that the "British people are responding nicely" to the recent mail-shot. He explained that he had decided that the time had come to make the British offshoot "more active", but that there were limits to the extent to which cancer could be dealt with "by throwing money at it". The British trustees, apart from Mr Salisbury himself, are Dr Colin Thomson and Professor Lord Tedder, also at St Andrews. □

AMPTE

British component fails

To the bitter disappointment of British space scientists, the United Kingdom's component of the AMPTE (Active Magnetospheric Particle Tracer Explorers) mission appears to have ended prematurely. The UK Subsatellite (UKS) is equipped to measure the particles and the magnetic and electric fields and waves encountered as it follows its West German companion satellite in an orbit through the magnetosphere. But on 16 January, the Rutherford Appleton Laboratory failed to detect a signal from UKS as it came over the horizon; subsequent efforts suggest that the fault lies in the spacecraft itself and is irredeemable.

The AMPTE mission achieved two major successes during earlier experiments, in which lithium and barium ions were released in the solar wind upstream of the Earth's magnetosphere. The German satellite, the Ion Release Module (IRM), and UKS were able to monitor the comet-like interactions which followed the releases (see *Nature* 10 January, p.90). The third AMPTE satellite, the US Charge Composition Explorer (CCE), is placed in an orbit within the radiation belts and, as well as continually monitoring the particle populations, is also hoped to detect any ions that might be injected into the belts following their release from IRM.

Earlier this month, British scientists were cock-a-hoop over the quality of data coming in from UKS. Following the disappearance of the signal, a search for the satellite has been carried out by the National Aeronautics and Space Administration's deep space network and other facilities. One possibility being considered was that the spacecraft had changed orbit due to an unplanned expulsion of gas. This is now ruled out. No signal has been detected and, after extensive checks of ground equipment, it has been concluded that the fault is on board.

According to a spokesman at the Rutherford Appleton Laboratory, the mission has achieved 70 per cent of its aims. One of those involved in the mission, David Southwood of Imperial College, London, expressed considerable frustration that UKS did not hold out until its orbit had precessed into the magnetotail, where a plasma release is planned. "Had it lasted until May or June it would have achieved 90 to 95 per cent of its aims" he said. "But the data sent back from the magnetopause [the boundary between the solar wind and the Earth's magnetosphere] are, in terms of time and angular resolution, the best achievable for some time to come, while the comet release experiments were unique."

Philip Campbell

NERC corporate plan

SIR — Your news item (*Nature* 10 January, p.91) on the corporate plan for the reorganization of the Natural Environment Research Council (NERC) is misleading. It implies that trades unions are now being consulted about the plan but many staff, certainly those scientists that the Institution of Professional Civil Servants represents in the British Geological Survey, regard the council's consultations with its unions as a sham. Most of the staff have not yet been allowed to see the document and they are anxious at the reported implications and angry at being refused sight of it. The few departmental union representatives who are privy to the

plan are forbidden to discuss it with their members.

NERC has been reported as saying that the reason for the secrecy is fear of misunderstanding but secrecy is far more likely to foster misunderstanding. As there is already widespread opposition to the plan, many believe the secrecy is really designed to minimize dissent before the plan is implemented.

NEIL AITKENHEAD
(Chairman)

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US space station experiments

SIR — In his article opposing European participation in the US space station project (*Nature* 1 November, p.11), Dr Erhard Keppler unduly minimizes the potential for biological investigation on board the proposed facility with his statement that interest in such work "... has greatly lost its attraction in the past few years". While it is true that fewer studies on the basic biological consequences of microgravity have recently appeared in the literature than one might expect, I believe that this is largely owing to a lack of proper facilities rather than to a shortage of interest.

An examination of earlier work published on vertebrate development in space reveals a depressing parade of experiments marred by lack of controls, launch delays, short flight times and constraints on cabin space and crew involvement, resulting in data of limited value. For example, the Soviets¹ flew developing frog larvae on board the Salyut-4 spacecraft and found changes that could be interpreted as degenerative in the vestibular sensory neuroepithelium of the inner ear, but as in the case of most other orbital biology work so far performed, 1g control centrifuge data are missing, so drawing a conclusion from the findings is risky. Similar experiments flown on Gemini² and Biosatellite³ flights also yielded unsatisfactory results. Good information on microgravitational effects on higher vertebrate development is especially scarce. A joint US-Soviet attempt to mate rats in orbit on Cosmos 1129⁴ failed to produce conception for reasons that are not clear (behaviour was apparently not recorded), while 60 incubating quail eggs on the same flight suffered developmental arrest when the humidifier broke down in mid-flight.

Rather than throwing up our hands and giving up, we should insist on proper facilities where careful long-term observations can be made directly by specialists. While the NASA/ESA Spacelab is a giant step in this direction (with some planned flights of the orbital laboratory devoted specifically to the life sciences), only a per-

manent space station offers the opportunity for continuing studies of animals and plants reproducing and developing in microgravity over multiple generations. This work has extremely important implications for the future of man in space and constitutes strong justification for going forward with the construction of a space station. Cooperation with European bioscience groups in such an endeavour would clearly benefit all.

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1. Vinnikov, Y.A. et al. *Arch. Anat. Histol. Embryol.* 70, no.1 (NASA TT F-16987, 1976).
2. Young, R.S. & Tremor, J.W. *Life Sci. Space Res.* 6, 87 (1968).
3. Edwards, B.F. *Life Sci. Space Res.* 7, 84 (1969).
4. Keefe, J.R. in *Final Reports of US Rat Experiments Flown on the Soviet Satellite Cosmos 1129* (NASA TM-81298, (1979)).

Nuclear winter

SIR — The National Research Council of the US Academy of Sciences has now joined others who have postulated severe winter conditions as an immediate consequence of any major nuclear war. So far such hypotheses have suggested that such conditions may be of limited duration — a matter of weeks or months — and appear to have concentrated on the direct loss of solar energy that would result from pollution of the atmosphere.

It would therefore be of interest to know whether any of the models so far used have taken account of predictable consequential energy losses — notably those attributable to the albedo effect of the snowfields that the nuclear winter would create and those that might result from the interruption of warm air and tidal flows into the higher latitudes of the Northern Hemisphere if the seas froze. On basic principles, it seems entirely possible that the aggregate of effects might well tip the scales to precipitate a major glaciation.

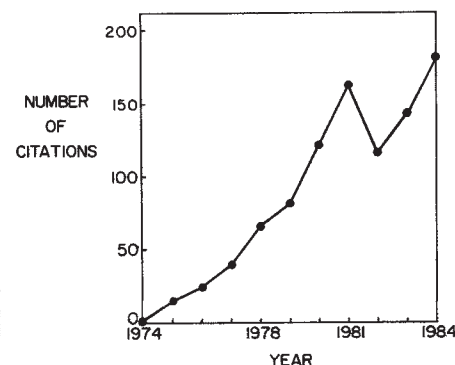
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Jerne still influential

SIR — Peter Newmark's use of the past tense in his brief review of Niels Jerne's Nobel Prize winning work (*Nature* 311, 601; 1984) is misleading. He writes that Jerne's network theory "was at its most influential about a decade ago". This seems to imply that the theory has since been discredited, and is no longer influential. It was however only a decade ago that Jerne's most famous network paper was published in the *Annales d'Immunologie* (Institut Pasteur), and it took some time for the concept to become popular. One measure of the degree to which a paper is influential is the extent to which it is cited. Figure 1 shows the number of citations of the above-mentioned paper for each year since then, according to the *Science Citation Index*. To judge by this criterion, that paper's degree of influence may not yet have peaked.

It is impractical to attempt to review in this letter the very considerable achievements of immune system network theory, as formulated by Jerne and subsequently modified and developed in more detail by others. Suffice it to say that Newmark's remarks clearly do not reflect current thinking of workers in the field.

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The number of citations of the paper "Toward a Network Theory of the Immune System" by N.K. Jerne, *Annls Immun., Inst. Pasteur, Paris* 125C, 373-389 (1974) for the years 1974-84. The number given for 1984 is twice the number of citations for January to June 1984.

Value-free science

SIR — Your correspondent Mark Diensdorf (*Nature* 10 January, p.92) throws at us the same superstitious rubbish we have been enduring for years. Is he (or anyone else) really unable to distinguish between the scientific (and value-free) statement "The lethal dose of cyanide is x gm/kilo of body weight" and the practical (and value-loaded) statement "I am going to poison my wife"?

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Antibiotics in animals

SIR — In his leading article of 4 October¹, Stephen Budiansky takes issue with some comments I made regarding a recent study² linking subtherapeutic use of antibiotics in animals with human disease³. While he obviously hears the clarion differently from me, it is important to clarify some of the facts that he presented. (Those addressed by A.H. Linton⁴ are not repeated here.)

Nobody will disagree with Mr Budiansky that *Salmonella* should not be in meat. These and other less pathogenic organisms will, however, continue to contaminate food products while they continue to be selected for in the agricultural environment. It is precisely their antibiotic resistance that assures this selection and reflects the use of antibiotics.

Mr Budiansky questions "whether animals are a significant generator of resistant human pathogens". It is becoming increasingly more evident that resistant *Salmonella* causing human disease in the United States originate in animals⁵. Moreover, once introduced into a human population, such as occurred in a hospital nursery⁶ or the outbreak described in the *New England Journal of Medicine*², human-to-human spread may also occur. But resistant non-pathogenic strains are also involved. Since transfer of resistance occurs readily among pathogens and non-pathogens, it is not necessary that "human pathogens interact with animal pathogens in order to acquire resistance". This plasmid exchange makes it very relevant to speak about the environmental pool of these resistance plasmids no matter what their host.

Mr Budiansky cites the article by Atkinson and Lorian⁷ as evidence that resistance is not being spread and that resistance in the environment has reached an "equilibrium". But these data are limited to hospitals which have always been a haven of antibiotic use and antibiotic resistance. Furthermore, the study did look "at the big picture", (5.8 million isolates from 242 hospitals in the United States from 1971 to 1972) with the consequence that one cannot relate the findings to any individual hospital or individual patient. In fact, the authors of the report admit that "local outbreaks of bacterial resistance do arise and many have serious consequences". Moreover, they report increased resistance to multiple antibiotics in *S. faecalis* and *S. epidermidis* and sustained high levels of resistance to common antibiotics among many of the strains tested. The fact that some (but not all) resistance has stayed the same and not decreased is hardly grounds for complacency. More importantly, these data do not take into account the new emergence of infectious agents such as multi-resistant pneumococci, penicillin-resistant gonococci, and ampicillin-resistant *Haemophilus influenzae*

which now appear in the community.

The microbial environment of animals and man is not separate. Consequently, one must look at the sources of these resistant bacteria and resistance factors in the "common" environment. To this viewer, for the reasons stated in my editorial, a major source in the United States is animals.

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1. Budiansky, S. *Nature* 311, 407 (1984).
2. Holmberg, S.D. et al. *New Engl. J. Med.* 311, 617-622 (1984).
3. Levy, S.B. *New Engl. J. Med.* 311, 663-665 (1984).
4. Linton, A.H. *Nature* 312, 96 (1984).
5. Holmberg, S.D., Wells, J.G. & Cohen, M.L. *Science* 225, 833-835 (1984).
6. Lyons, R.W. et al. *J. Am. med. Ass.* 243, 546-547 (1980).
7. Atkinson, B.A. & Lorian, V.J. *Clin. Microb.* 20, 791-796 (1984).

Stephen Budiansky replies:

Levy seems to suggest that feeding farm animals antibiotics makes contamination of meat by salmonella more likely. In fact, several studies address this very question and reach the opposite conclusion¹⁻².

But in any event, the burden of my argument was that salmonella is simply the

wrong example to be looking at. Antibiotic resistance is presumably of concern because it is making the treatment of bacterial infections more difficult. Since antibiotics are not normally used to treat salmonellosis, it really does not matter whether the salmonella that is making people sick is resistant or not, or of animal origin or not. Furthermore, salmonella is one of the few pathogens common to farm animals and humans; that animal feeds may be responsible for resistant salmonella does not mean that they are responsible for resistant strains of other, strictly human, pathogens that are one step removed from animal guts.

Indeed it seems unlikely that the blame for ampicillin-resistant *H. influenzae* can be laid to animal feeds, since ampicillin is not used in feeds. And why postulate a subtle origin of penicillin-resistant gonorrhoea, involving multiple transfers of resistance plasmids between bacterial species, when we have staring in our faces the blatant bombardment of humans (and especially those humans with gonorrhoea) by penicillin for the last 40 years.

1. Layton, H.W. et al. *Vet. Med. Bull.* 22, 461-472 (1975).
2. Evangelisti, D.G. et al. *Antimicrob. Agents Chemother.* 8, 664-672 (1975).

Too much hype

SIR — An aggressive publicity campaign has been launched to promote Immunovir (or Isoprinosine), recently approved in Great Britain^{1,2}. The campaign has even found some echo in the columns of *Nature*³.

This is hardly surprising, since this drug is already better known from its promotion rather than its results. For example, in 1982, the US drugstore bookstalls hyped the drug with a paperback, which not only claimed a cure for herpes but also accused the Food and Drug Administration (FDA) of preventing herpes victims from using and benefiting from the miraculous substance⁴. Although the author did not support his claims with any scientific publications, the book claimed him to be "an authority on herpes".

It is even more curious that reports describing the ineffectiveness of Isoprinosine⁵⁻⁷ seem to have been ignored on the occasion of its British premiere, as was also the fact that despite the commercialization of Isoprinosine in France, Germany, Italy and Spain for several years, the number of herpes patients has not decreased there. The purpose of this communication, however, is not to add data based on experience with this drug showing that Isoprinosine clearly has no beneficial effect on herpes patients, but rather, to question the practices of pharmaceutical companies and of the media.

It is therefore incredible to read in the *Sunday Times*: "A pill for AIDS" is available, "but it may come too late"¹. Or in the *Financial Times* that: "Immunovir may

also be able to prevent AIDS, a fatal breakdown of the immune system"². Last but not least, there is the statement: "The drug Immunovir is the first in a new class of drugs which appear to have very promising potential in treating and preventing viral diseases ranging from influenza to shingles to AIDS and to some kinds of cancers"².

It is time to ensure that responsible lay publications prevent the exploitation of the public in such ways. In an era of proliferation of regulatory bodies and ethics committees whose role and motivations are not always obvious, it is unfortunate to have to suggest yet another body to impose restrictions on scientists and journalists concerning the publicity of potential "breakthroughs". But, if it is unacceptable that research hopes are wildly publicized (for more often than not false hopes are more damaging for the public than patience), only self-discipline by scientists and journalists will eradicate the sensational.

Certainly, those who have vested interests in pharmaceutical companies, politicians promoting their re-election or scientists promoting their career and their laboratory budget through the lay press, will reject such limitations, but the majority would accept them with relief.

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1. Deer, B. *Sunday Times* 14 October, 9 (1984).
2. Rapoport, C. *Financial Times* 10 October, 7 (1984).
3. Wenz, C. *Nature* 311, 404 (1984).
4. Wickett, W.H. Jr M.D. *Herpes: Cause and Control* (Pinnacle, New York, 1982).
5. Bierman, S.T. *Semin. Derm.*, 3, 106-112 (1984).
6. Saurat, J.H. *Annls. Derm. Vener.*, 111, 71 (1984).
7. Kalimo, K.O.K. et al. *Archs. Derm.* 119, 463-467 (1983).

Radiation casualties in a nuclear war

from Patricia Lindop, Joseph Rotblat & Philip Webber

Calculations of the effects of nuclear explosions over London suggest that the proportion and absolute number of radiation fatalities are higher than previously estimated. They are sensitive to the value of LD_{50} assumed for people, but the number of total casualties, deaths plus injuries, is practically independent of the LD_{50} .

THE several recent calculations of the numbers of casualties likely in a nuclear war differ widely in their estimates of casualties caused by radiation, both absolutely and relative to other major causes of death or injury, the blast wave and the heat pulse. The ratio of radiation to blast deaths has been variously estimated as 6 per cent¹ or 86 per cent². Part of the discrepancy arises from the different assumptions made about the protection afforded by buildings, part from the relationship assumed between the radiation exposure of individuals and the risk of mortality.

In calculations of the consequences of nuclear war, the choice of a value for LD_{50} , the radiation dose causing 50 per cent mortality in human beings, seems to be somewhat arbitrary. Indeed, there are very few data on which to base accurate estimates. Such information as there is derives from radiation accidents in peacetime, with victims receiving first-rate medical care. The experience in Hiroshima and Nagasaki has not so far been used in this respect, but clearly in the aftermath of a nuclear war the radiation mortality would be much greater than in peacetime.

Here we consider two cases — a single one-megaton bomb in a ground-burst on a major city (we have chosen London so as to work with a known population distribution) and the explosion of several thermonuclear weapons on the periphery of a major city. The first case is probably an unrealistic representation of nuclear war, but is simple to calculate and easily translated to other circumstances. The second is the London component of the 1980 'Square Leg' exercise of the British Home Office. We also consider the effect of sublethal exposures in the two cases.

Single bomb

We first assume that a one-megaton bomb (half-fission, half-fusion) is exploded 580 metres above the centre of London. For such a detonation, the fireball would touch the ground, giving rise to local fall-out. Before calculating the effects of the latter, the deaths due to the heat and blast waves in a given area have to be subtracted. Those due to the initial neutrons and γ rays can be ignored, since they occur within the lethal area of the blast effect. Using a procedure suggested by the US Office of Technology Assessment³, we have calculated that the total number of fatalities from heat and blast would amount to 560,000 out of a London population of 7 million.

For the estimate of fall-out casualties, we have assumed a steady southerly wind blowing at 24 kilometres per hour, and zero wind shear. We have ignored the effects from the inhalation and ingestion of radioactive materials, but have considered only the γ rays from the fission products after their deposition on the ground. A terrain shielding factor of 0.7 was applied to allow for roughness of the surface. Under the postulated idealized atmospheric conditions, the contours for constant dose-rate could be assumed to be ellipses with dimensions deduced from the formulae given by Glasstone and Dolan⁴. In combination with the population densities in the individual London boroughs, we were thus able to calculate the numbers of people receiving a given dose in a specified time under stipulated exposure conditions. From the sigmoid-shaped curves relating mortality to radiation dose, we then calculated the number of fatalities for the various parameters in our scenario.

We have chosen four values of the LD_{50} , to cover the likely range, namely 3, 4, 6 and 8 gray, these being tissue doses at the surface of the body. For each LD_{50} value we calculated the casualties for four values of the protection factor (PF) — 1, 5, 10 and 20. The first of these refers to the case in which people remain in the open during the whole period of exposure. In war scenarios described in the literature, a protection factor of 5 is often used as an average value for city populations. We have also calculated the effect of receiving the exposure in one day and over 7 days. The latter is the period during which the accumulated dose is nearly equal to the infinity dose, although if people in shelters with a high PF emerged after 7 days, they could still accumulate a large dose if they subsequently remained in the open. A one-day exposure is of relevance in conjunction with a PF of 1; if the bomb were part of a surprise attack, many survivors of the heat and blast effects would be disoriented — some would try to get home, others would try to leave the city, but it might take a day to find an uncontaminated area or a shelter.

Table 1 contains the results of the calculation of radiation fatalities following a 7-day exposure. The numbers have been rounded off to the nearest 10,000; greater precision is not warranted. There is more than a 10-fold difference in the number of fatalities between the lowest and highest values of the parameters used. But the main

point to note is the large absolute number of deaths. At the conditions most often assumed in the literature, $LD_{50} = 4$ Gy and $PF = 5$, the radiation deaths come out to be the same as the total from blast and heat.

Operation Square Leg

In September 1980, several British government departments conducted an exercise, code-named Operation Square Leg, as part of the NATO Crusader exercise, to evaluate the effects of a nuclear attack on Britain. In this exercise, 125 nuclear weapons with a total yield of about 200 megatons were assumed to have been exploded. We analysed the effects of four of these bombs exploded on three targets, all within the Greater London Council (GLC) area but on its periphery. Three of the weapons were assumed to have detonated at ground level, namely at Heathrow (1 Mt), Brentford (2 Mt) and Croydon (3 Mt). Heathrow was also subjected to a 2-Mt weapon detonated at a height of 3.6 km, producing heat and blast casualties, but no local fall-out (Fig. 1).

Out of the initial London population, 1.08 million deaths from blast and 0.27 million from heat were subtracted before calculating the effects of radioactive fall-out. The atmospheric conditions were assumed to be the same as in the case of a single bomb. The calculation of radiation casualties was carried out, using the same methods as described earlier, for LD_{50} values of 4 and 8 Gy, protection factors of 1 and 10 and exposure duration of 7 days.

The calculated numbers of radiation fatalities are: at $PF = 1$, 3.75 and 3.26 million for LD_{50} values of 4 and 8 Gy. The corresponding values at $PF = 10$ are 1.91 and 1.09 million. Again, the large absolute number of radiation deaths is notable. Even though one of the four bombs did not contribute to the fall-out, the interpolated value of radiation fatalities at the typical

Table 1 Radiation fatalities after 7-day exposure (thousands)

LD_{50} (gray)	Protection factor			
	1	5	10	20
3	1,870	680	420	270
4	1,580	550	340	210
6	1,270	420	270	160
8	1,050	340	210	140

The numbers in this 4×4 matrix fit a simple empirical formula: $N = 4 \times 10^6 (PD)^{-2/3}$, where N is the number of radiation deaths, P the protection factor and D the LD_{50} value in gray.

Big machines make big science

Britain's problem, whether to pull out of high-energy physics, would be solved if there were a moratorium on machine-building. Astronomy is in the same case.

Two or three months from now, the British research enterprise will have gone through its biggest upheaval since the Second World War. Then four successive governments (two Attlee, one Churchill and one Eden) allowed that the great wartime successes (radar, jet engines and even nuclear fission — not, in the end, very British) should be followed by deliberate support for research laboratories on a scale and in a manner different from the casual arrangements of the 1930s, when penicillin was discovered by accident and, by a similar kind of accident, almost let slip. There is much in the view that what is happening in Britain now is peculiarly British. But there is also something to be learned elsewhere from the present dilemma.

Most civil research, certainly most academic research, in Britain is paid for by the government, through the medium of four supposedly autonomous research councils. (There is a fifth, responsible for the social sciences.) Forty years ago, however, there was no mechanism for the support of academic research in general. The agricultural and medical research councils existed to encourage the application of science in their special fields, the natural environment research council had not been invented, the notion that university laboratories were an important source of innovation was acknowledged but not thought to require special intervention and the sole source of financial support for untied research was the Department of Scientific and Industrial Research, whose terms of reference were to make Britain prosperous.

For many years after 1945, the chief sources of support for science were agencies not very different from those still supporting science in other places. The British Ministry of Supply, an early version of a defence procurement agency, founded both the post-war aircraft industry in Britain and also the atomic energy industry, creating in the process the nucleus of what has now become the high-energy physics community. In those days, the Royal Greenwich Observatory was supported by the Admiralty.

In a curious way, the question now for decision by the research councils reopens the questions decided without much thought forty years ago. Now, the Science and Engineering Research Council, having been told firmly by the Treasury that it can look for no help this year in paying its sub-

scription to CERN (the European Organization for Nuclear Research), is being forced to ask whether Britain can continue spending some £70 million a year on high-energy physics. By the spring a decision whether or not to pull out at the end of this year will have had to be made.

What should it be? Everybody agrees that this would be a rotten time for a country such as Britain, with such traditions, to pull out of high-energy physics. For is it not exciting that the predictions of the electro-weak theory should have been confirmed in the past year or so and that the prospect of further adventure in fields such as super-symmetry should have been opened? That Britain, in whose universities the whole field of nuclear physics was invented (principally by Rutherford, at Manchester and then Cambridge), should have to decide to have no more to do with it would be acutely galling. Even physicists who are not nuclear physicists, or scientists in general, agree that such an outcome would be a misfortune. Some, however, hold that following the line dictated by the affection would rob more orthodox fields of science of the support they need, and would reluctantly cut adrift from what sentiment dictates.

It is less often remarked that there are other fields of scientific work in which much the same conditions apply. Britain has become an important contributor to international research in astronomy not because of natural advantages but for the opposite reason, by a kind of perverse insistence on doing well in spite of natural disadvantages. To be sure there is again a strong tradition, going back to Newton and Herschel, but more recently represented by theoreticians such as Eddington and Hoyle, of people who have been able to construct imaginative glimpses of what the Universe is like. Only in the late 1950s did British astronomy seek to compete observationally with, say the Californians. Only in the past few years, in Australia, Hawaii and, in due course, on Tenerife, has the competition showed signs of being successful.

The two fields, high-energy physics and observational astronomy, have much in common. In each of them, purpose-built instruments can be powerful stimulants of people's curiosity. Indeed, an expensive instrument may create an economic need that further resources should be spent, both on ancillary equipment and on training people, so as to win the full benefits of the original investment. This is the spirit, in the

late 1960s, in which the British high-energy physics community kept urging on anybody prepared to listen the need for further investments beyond the cost of particle accelerators (at CERN but also domestically). More recently, the astronomers have made the same case, more quietly but as successfully; more than a third of British universities now teach astronomy to graduate students, the others would give their eye-teeth to afford to do so. The government which ultimately pays the bill regrets that more effort is not being spent on research to make industry competitive.

In high-energy physics, it is often said that after the next machine is built, the time will have come to put high-energy physics on an international basis, with interested governments clubbing together to build a machine that none of them could separately afford. That way, the argument goes, it will be possible to maintain the momentum of discovery in spite of the gigantic cost. But in this field, as in astronomy, there is no compelling reason why the next discovery should be made tomorrow rather than, say, the day after tomorrow. Or, more accurately, the urgency of the need to know precisely what symmetry groups govern the relationships between elementary particles, or whether the distance-scale of the Universe corresponds to an age of 10,000 million years or twice as much, is academic compared with the need somehow to ensure that academics have the resources with which to answer questions of that kind.

A few morals follow simply. First, it will of course be tragic for many people, but also a break with honourable tradition, if British science has to retreat from high-energy physics, astronomy, either or both. But it may be necessary. And it should not be astronomers or high-energy physicists who decide. Second, since there is already a sense in which these enterprises are international, may it not be worthwhile negotiating not an international agreement for the common building of machines, but international agreements to limit investment in fields where curiosity can be kept waiting? The trouble is that building machines, however expensive, has become too simple. The Large Space Telescope (see p. 337) will settle the issue of the distance-scale in a few days' observation, and will then for twenty years consume the efforts of an army of astronomers, many not yet created. A moratorium on building other telescopes would be in the public interest.

John Maddox

Langmuir-Blodgett films

Organic monolayer imaged

from R.H. Tredgold

THE Langmuir-Blodgett technique has recently aroused considerable interest as a way of forming highly ordered monolayers and multilayers of amphiphilic molecules. With their paper on page 382 of this issue, J.R. Fryer, R.A. Hann and B.L. Eyres establish, for the first time, the structure of an organic monolayer formed by the technique¹. This represents a considerable technical feat and a milestone in an interesting history.

Early work on Langmuir-Blodgett films was confined to fatty acids but recently films have been formed from a wide variety of materials including amphiphilic porphyrin derivatives and copolymers bearing both hydrophilic and hydrophobic side groups in regular alternation. It is possible to synthesize a variety of amphiphilic porphyrin derivatives starting from naturally-occurring materials which have a built-in asymmetry but, because it is extremely difficult to synthesize a pure asymmetric phthalocyanine, it has not been possible to produce an amphiphilic derivative. Remarkably, however, Fryer *et al.* have succeeded in forming a Langmuir-Blodgett monolayer from a symmetric phthalocyanine derivative. Although the layer spacing of Langmuir-Blodgett films can be found from low angle X-ray diffraction, it is much more difficult to find the arrangement of molecules within a layer because some support is necessary and the supporting material is likely to confuse electron diffraction patterns. Such thin films also have a tendency to overheat and disintegrate under the influence of an electron beam. It is thus all the more remarkable that the structure of a monolayer has now been established.

The history of the technique is also of considerable interest. 'Spreading oil on troubled waters' is a technique known since ancient times, and is referred to by Pliny the Elder. The first scientific study to be published was that of Benjamin Franklin². In the early 1890s Agnes Pockels published two short papers in *Nature*^{3,4} describing her studies of monolayers of various materials, including stearic acid, spread on water. Considering that Agnes Pockels was limited in apparatus to what was available in the family kitchen, her achievements are truly remarkable. She established most of the basic technology of what are now known, rather

unfairly, as Langmuir films and obtained a value of about 2.3 nm for the length of a stearic acid molecule, in reasonable agreement with the actual value of 2.5 nm. In 1917, Langmuir published some of his experiments and in 1920 he described the transfer of a fatty acid monolayer to a solid substrate⁵, acknowledging Miss Katherine Blodgett "for carrying out most of the experimental work". In 1935, Katherine Blodgett published a lengthy paper⁶ describing the formation of multilayers and their characterization; such layers are now

invariably known as Langmuir-Blodgett or LB films. The next significant development was the use of Langmuir-Blodgett films to study the interaction of dye molecules by Kuhn and his collaborators during the 1960s and 1970s⁷.

Subsequently, interest in Langmuir-Blodgett films has increased. Apart from their intrinsic scientific fascination, they have considerable technical potential in non-linear optical wave guides, high resolution electron beam resists, sensitive gas detectors and high efficiency solar diodes. Two special editions of *Thin Solid Films*^{8,9} devoted to this topic give a general idea of modern developments and interests. □

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Neuronal development

Serotonin-containing cell charged with growth cone arrests

from I.A. Meinertzhagen

IF THE developmental neurobiologist would dare re-write Sherrington's metaphor for the brain as "an enchanted loom where millions of flashing shuttles weave a dissolving pattern"¹, then the shuttles might very well be the growth cones, the motile organelles which spin out the tips of all growing neurites, so weaving the fabric of the developing nervous system. Knowledge of factors which determine the course of these organelles or which influence the decisions they make during their progress is therefore essential to any understanding of the assembly of developing neural circuits. Recent studies, *in vivo* and *in vitro*, indicate the nature of some of these signals. Nerve growth factor (NGF)², the surfaces of other cells³ and of the extracellular matrix⁴, and electric fields⁵ have all been shown to have direct effects on growth cones. A recent report in *Science* puts forward neurotransmitters as the latest candidates for growth cone signals⁶.

Individual identified adult neurones of the snail *Helisoma*, when cultured *in vitro*, form growth cones and extend neurites. P. G. Haydon *et al.*⁶ report that serotonin (5-hydroxytryptamine) selectively inhibits the extension of exploratory filopodia from growth cones of the neurone known as cell 19 in the buccal ganglion. As a consequence, outgrowth of neurites is arrested, an effect that is reversible. The action of serotonin is localized to the growth cone, as it can be produced by focal application from a micropipette placed on the surface of growth cones which have been isolated from their neurites. These results reveal for the first time that a substance hitherto known only as a neurotransmitter in the nervous system also has a morphogenetic action, *in vitro* at least. The only previously

known chemotropic agent that directly influences neurite extension at the growth cone is NGF, which increases filopodial exploration and accelerates the extension of neurites from dorsal root ganglion cells of embryonic chicks. These actions are accompanied by progressive turning of the growth cones toward the NGF source.

Inhibition of growth cone motility by serotonin has implications for the formation of synapses between neurones. When a *Helisoma* buccal ganglion cell 5 is axotomized *in situ*, it grows extensive neurites⁷. It also develops stable electrical coupling with other cells 5 and transient coupling with other identified neurones. It does not, however, establish coupling with cell 19 and other buccal protractor motor neurones⁷. The coupling, presumably mediated by gap junction contacts, is found only after axotomy and not in the circuitry of the normal buccal ganglion. When individual neurones are isolated from the adult ganglion and placed in dispersed-cell culture, their behaviour resembles that following axotomy: they undergo neurite growth and become electrically coupled⁸. In this case, however, cells 19 and 5 become coupled, apparently because they are concurrently producing neurites. When such neurones are added to an established cell culture, they may form elaborate co-extensive neurites with the older cells but nevertheless fail to become coupled to them, suggesting that competence to couple electrically is a manifestation of simultaneous growth⁸. Haydon *et al.*⁶ reason that if serotonin inhibits such growth, it might also inhibit the appearance of coupling. They show that serotonin applied to a mixed culture of cells 19 and 5 both selectively inhibits neurite extension in cell 19

1. Fryer, J. R., Hann, R. A. & Eyres, B. L. *Nature* 313, 382 (1985).

2. Franklin, B. *Phil. Trans. R. Soc.* 64, 445 (1774).

3. Pockels, A. *Nature* 43, 437 (1891).

4. Pockels, A. *Nature* 46, 418 (1892).

5. Langmuir, I. *Trans. Faraday Soc.* 15, 62 (1920).

6. Blodgett, K. B. *J. Am. Chem. Soc.* 57, 107 (1935).

7. Kuhn, H. *J. Photochem.* 10, 111 (1979).

8. *Thin Solid Films* 68 (1980).

9. *Thin Solid Films* 99 (1983).

and prevents the establishment of the expected coupling with cell 5. On the other hand, a pair of cells 5 in the same culture do become electrically coupled⁶. Thus, cell 5 is capable of establishing electrical coupling in the presence of 10^{-6} M serotonin, whereas cell 19 is not.

Clearly the importance of these findings depends upon the specificity of the chemotrope's action. Selectivity of cellular response has already been demonstrated, with cells 19 responding to serotonin at concentrations of 10^{-7} M and cells 5 not responding at all to concentrations up to 500-fold greater (5×10^{-5} M). Do other neurotransmitters have a similar action? If so, do they affect the same neurones; or is the growth of different neurone classes coded to respond to different neurotransmitters? Answers to these questions are needed before such intriguing observations can be said to reveal an action that is specific to either a class of cell or a type of neurotransmitter.

But is serotonin really involved in neuronal development? Although the authors are curiously silent on this point, further light can be shed on the unwritten significance of their findings. On either side of the brain in various gastropod molluscs is a large, unique neurone which projects to the buccal ganglion and often contains serotonin. In *Helisoma*, this cell is known as C1 (ref. 9); its axon and terminals are the only structures in the buccal ganglion that react with antibody to serotonin (unpublished observations cited in ref. 10). Electrical stimulation of C1 can initiate the buccal ganglion-generated feeding rhythm, an action that is mimicked by application of serotonin at 5×10^{-7} M to the whole ganglion⁹. Granzow and Kater⁹ have taken this to imply that serotonin is the

putative neurotransmitter of C1. Two pieces of unpublished evidence (J. Goldberg and S.B. Kater, personal communication) suggest that this cell similarly is a source of serotonin during embryonic development. Firstly, the cytotoxic transmitter analogue 5,7-dihydroxytryptamine, which acts selectively on serotonergic neurones, temporarily depletes serotonin when applied to embryos. If it is used at a stage when cell 19 is extending neurites, changes in the morphology of the adult cells 19 result, suggesting that these cells in the embryo respond to the same signals *in vivo* as do their adult counterparts *in vitro*. Secondly, immunocytochemical staining indicates that C1 is present and charged with transmitter at this same embryonic stage, indicating the possibility that C1 is the source of serotonin. If C1 is charged with serotonin at a time in embryonic development when the growth of cell 19 neurites is sensitive to it, all that remains to be demonstrated is that serotonin is actually released at both the time and the concentration necessary to achieve the desired morphogenetic effect. This is a tall order, but whoever fills it will write a new chapter in neural development. □

1. Sherrington, C.S. *Man on his Nature* 2nd edn (Cambridge University Press, 1951).
2. Gundersen, R. & Barrett, J. *J. Cell Biol.* **87**, 546 (1980).
3. Goodman, C.S. *et al. Science* **225**, 1271 (1984).
4. Carbonetto, S. *Trends Neurosci.* **7**, 382 (1984).
5. Patel, N. & Poo, M.M. *J. Neurosci.* **2**, 483 (1982).
6. Haydon, P.G., McCobb, D.P. & Kater, S.B. *Science* **226**, 561 (1984).
7. Bulloch, A. & Kater, S. *J. Neurophysiol.* **48**, 569 (1982).
8. Hadley, R.D. & Kater, S.B. *J. Neurosci.* **3**, 924 (1983).
9. Granzow, B. & Kater, S.B. *Neuroscience* **2**, 1049 (1977).
10. Trimble, D.L., Barker, D.L. & Bullard, B.J. *J. Neurobiol.* **15**, 27 (1984).

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Geophysics

Global sea level trends

From J.E. Hansen

GLOBAL sea level is rising but at a rate, of about 1-2 mm per year or 10-20 cm per century, that is difficult to measure accurately. Several mechanisms may contribute to the net increase but it is difficult to distinguish their individual contributions. These conclusions are emphasized by Barnett¹ and in several other recently published papers.

Sea level trends are estimated from records of tide gauge stations, which are spread very inhomogeneously around global shorelines. The trends recorded at different stations range widely from an increase in sea level of one metre per century in Louisiana to a fall of one metre per century in parts of Scandinavia. The largest trends result from vertical shoreline movements (caused by river delta sedimentation in Louisiana and post-glacial crustal uplift in Scandinavia), but many factors introduce

local variability, including changes of ocean currents, ocean heat content, atmospheric pressure, atmospheric wind patterns, local vulcanism and tectonism.

Some of the local factors are unimportant when averaged over a period of several decades; others can be identified at individual recording stations, data from which can then be excluded from global analyses. Even so, Barnett stresses that the global estimate he obtains is still uncertain by at least 50 per cent, because variations of that size emerge from different ways of averaging over the globe. Barnett's principal result — a 14 cm rise in global sea level in the past century and a rate of 23 cm per century in the past 50 years — is consistent with the results of previous investigators. This is not surprising because, as Barnett notes, most investigators have worked with essentially the same data set.

Trends in sea level have practical significance. The current rise of approximately 30 cm per century along much of the US east coast, which is still subsiding because of the flow of material in the Earth's mantle towards the Hudson Bay region of post-glacial rebound, leads shoreline geologists and engineers to recommend against a strategy of installing stabilization structures to arrest the erosion of beaches. The rising sea level, combined with natural shoreline fluctuations and often with erosion caused by the 'stabilization' structures, is making it essential for coastal planners to take into account a shifting and generally retreating shoreline. Present sea level trends have an even more dramatic effect in Louisiana, where a single county (Terrebonne Parish) is losing 20 km² (6,000 acres) of land per year to the encroaching sea.

But the main reason for the growing interest in sea level^{2,3} is the concern that global warming, due to increasing atmospheric CO₂ and trace gases, may lead to partial disintegration of the polar ice sheets and a subsequent large increase in the rate of rise in sea level. If entirely melted, the West Antarctic ice sheet, thought to be the most vulnerable since it rests on bedrock well below sea level, would raise global sea level by about 6 m. Greenland contains a similar amount of ice and East Antarctica has an order of magnitude more. Glaciologists estimate that West Antarctica could conceivably disintegrate in a time as brief as 200–500 years^{2,4}. Even without any net melting from the other ice sheets, such a fast response would have enormous practical impact, with the sea level on almost all coastlines rising at a mean rate of one metre or more per century.

Does the current increase in global sea level of 10-20 cm per century mean that the ice sheets have already begun to disintegrate? No. First of all, part of the rise probably results from the continuing influence of post-glacial isostatic disequilibrium^{5,6}, the high viscosity of the Earth's mantle implying that coastline elevations are still adjusting to the shift of water mass from continent to ocean which occurred with the melting of Würm-Wisconsin ice in the Northern Hemisphere. Models for crustal deformation⁶ suggest that this could account for a rise of 2-5 cm per century.

Thus, of the nominal 14 cm rise in sea level per century reported by Barnett¹, only about 10 cm is due to an increase in ocean water volume. Gornitz *et al.*⁷ estimate that a rise of 3-6 cm may be due to thermal expansion of ocean water in response to the 0.5°C global surface warming of the past century⁸. This warming is probably also responsible for the mean retreat of mountain glaciers in the past century, which Meier⁹ estimates to contribute an additional rise in sea level of 2-5 cm.

In view of the large recorded variations in sea level rises and in view of several other processes (such as ground water discharge and dam construction) which could account for changes in ocean water volume of 1-3

cm per century, we can conclude only that polar ice sheets must have been in approximate mass balance (± 10 cm of sea level) during the past century. This conclusion does not reduce the importance of understanding the mass balance of ice sheets, which is essential if we are to anticipate the effects of sea level of the 'greenhouse' warming predicated for coming decades. The fact that thermal expansion of ocean water and the retreat of mountain glaciers contributes significantly to sea level rise will make any future disintegration of ice sheets all the more important.

The task of analysing sea level and its relation to climate trends is a good example of an emerging class of 'global habitability' problems that many scientists expect to become increasingly important as man's impact on the global environment continues to grow. The scientific challenge is to develop a quantitative understanding of environmental changes. This requires us first to distinguish long-term trends, both man-made and natural, from short-term fluctuations. To meet this challenge will require the definition, implementation and maintenance of observation programmes on the time scale of decades.

Progress in understanding the relation between sea level and climate will depend especially on accurate monitoring of the volume of the major ice sheets. This could

be achieved with precise satellite altimetry using existing technology^{10,11}, the chief requirement being a polar-orbiting platform. Measurement of the thermal expansion of ocean water will require repeated hydrographic sections of the major ocean basins, necessitating regular cruises to monitor the ocean's temperatures, density and salinity structure. It should also be possible to determine net sea-level trends more accurately by means of additional well-chosen tide stations, mainly in the Southern Hemisphere. □

1. Barnett, T.P. *J. geophys. Res.* **89**, 7980 (1984).
2. Barth, M.C. & Titus J.G. (eds) *Greenhouse Effect and Sea Level Rise* (Van Nostrand Reinhold, New York, 1984).
3. *Changing Climate* (National Academy Press, Washington, 1983).
4. Bentley, C.R. in *Climate Process and Climate Sensitivity* (eds Hansen, J. & Takahashi T., American Geophysical Union, Washington, 1984).
5. Clark, J.A., Farrell, W.E. & Peltier, W.R. *Quat. Res.* **9**, 265 (1978).
6. Lambeck, K. & Nakiboglu, S.M. *Geophys. Res. Lett.* **11**, 959 (1984).
7. Gornitz, V., Lebedeff, S. & Hansen, J. *Science* **215**, 1611 (1982).
8. Hansen, J. *et al. Science* **213**, 957 (1981).
9. Meier, M.F. *Science* **226**, 1418 (1984).
10. Zwally, H.J., Bindshadler, R.A., Brenner A.C., Martin, T.V. & Thomas, R.H. *J. geophys. Res.* **88**, 1589.
11. Bufton, J.L., Robinson, J.E., Feniano, M.D. & Flatow, F.S. *IEEE Trans. Geosci. Rem. Sens.* **GE20**, 544 (1984).

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Neurobiology

Molecular tinkering that tailor the acetylcholine receptor

from Charles F. Stevens

ON PAGE 364 of this issue, the Kyoto laboratory of S. Numa reports another dramatic contribution to understanding the molecular basis of neurotransmission. By the technique of site-directed mutagenesis (which I have briefly explained in *Trends in Neurosciences* **7**, 306; 1984), Mashina *et al.* have made deliberate alterations in the structure of one of the subunits of the acetylcholine receptor (AChR) to test the functions of its parts. As usual, Numa's laboratory has done it grandly: two and a half dozen deletions and substitutions have been made and their effects analyzed.

First, some background. The AChR is one of several integral membrane proteins that are responsible for the electrical activity of the nervous system. Each of the proteins forms a channel that is selectively permeable to ions; the job of AChR is to transduce the signals delivered by acetylcholine (released from a presynaptic nerve terminal) into increased cation permeability of the postsynaptic membrane, in which the receptor molecules are embedded. The receptor is composed of four different membrane-spanning subunits (alpha, beta, gamma, and delta); a single AChR contains two copies of alpha, the subunit that binds

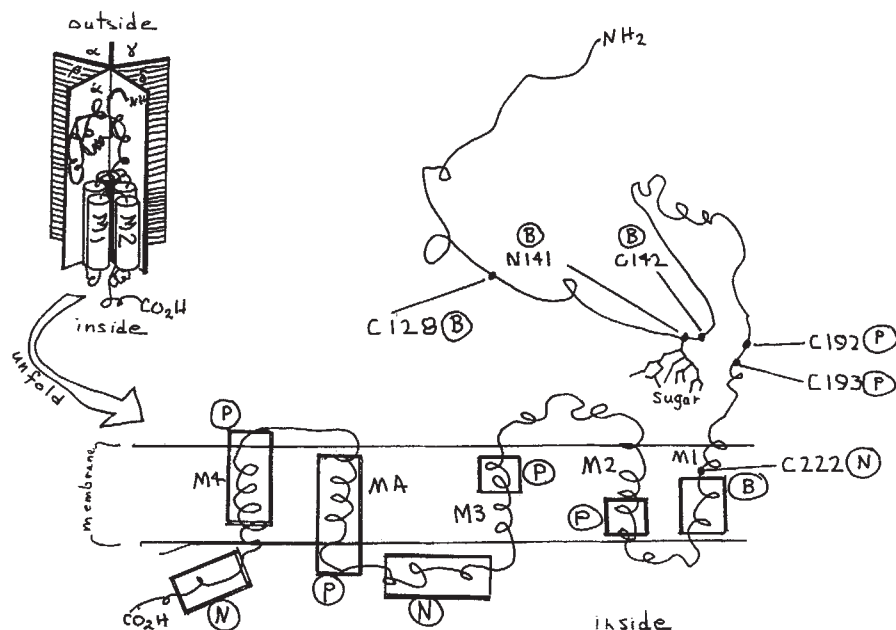
acetylcholine, and one copy each of the others. Although each subunit type has a distinct amino acid sequence and is coded by a separate gene, they all share the basic structure that is illustrated for alpha in the bottom section of the figure. The amino-terminal half of the receptor is extracellular. The carboxy-terminal half, according to the models of Finer-Moore and Stroud (*Proc. natn. Acad. Sci. U.S.A.* **81**, 155; 1984) and Guy (*Biophys. J.* **45**, 249; 1984) passes five times across the membrane. Four of these membrane-spanning segments (denoted M1 to M4 in the figure) are hydrophobic, and one (MA) is amphipathic; that is, it has charged side chains of amino acids displayed along one face with the other faces being hydrophobic. Both models postulate that each of the five subunits contributes an amphipathic helix to form the charge-lined walls of a water-filled pore through which ions pass. The hydrophobic helices are packed around this pore to stabilize the structure.

The alpha subunit has a binding site for acetylcholine that Karlin's laboratory (Kao, P.N. *et al. J. biol. Chem.* **259**, 11662; 1984) has recently shown to be near a particular cysteine (C192) that participates in a disul-

phide bond with one of the other three cysteines in the extracellular portion of the protein. It also has a single site for N-glycosylation at asparagine 141 (N141).

For AChR to transduce the acetylcholine signal into the response of increased cation permeability, it employs three functions: acetylcholine binding, gating (that is, opening the door that covers the pore), and ion permeation and selectivity (that is, the flow of certain cations like sodium, but not anions, through the pore). These three functions are well studied physiologically, but it has not been known how they arise from the molecular structure of the receptor. The accomplishment of Mashina *et al.* is to provide, at least in a preliminary way, an assignment of these functions to specific regions of the alpha subunit of AChR. After site-directed mutagenesis, the altered alpha subunit cDNA was used to make a corresponding mRNA, which was injected into frog oocytes together with normal mRNAs for the other three subunit types. The injected oocytes synthesize and assemble AChR, which is inserted into the surface membrane of the oocyte where its function can be studied. For each alteration, some of which deleted regions of the alpha subunit and some of which substituted one amino acid for another, the total amount of modified AChR made by the oocyte was determined with antibodies. In addition, the function of the AChR was assessed by measuring both the binding of ligands specific to the acetylcholine binding site (α -bungarotoxin and carbamyl choline) and gating combined with permeation in terms of the current flow produced by acetylcholine application.

As illustrated in the figure, various deletions were made in each of the five postulated membrane-spanning regions and in the cytoplasmic region between MA and M4; furthermore, each of the five cysteines of the subunit was separately replaced by a serine, and aspartic acid was substituted for an asparagine (N141). Most of the results fall into one of three categories: no effect observed (N in the figure); binding still intact but no gating or permeation (P); neither binding nor permeation functions intact (B). In some instances, decreased quantities of protein are detected, probably because one or more subunits fail to fold and assemble. The antibody-detection methods used cannot distinguish between cytoplasmic subunits and those displayed on the cell surface. In general, the findings support the models published by Finer-Moore and Stroud and by Guy for the membrane-spanning portions (although there may be a difficulty interpreting one large MA deletion) and are consistent with the location of the acetylcholine-binding site near C192 as suggested by Kao *et al.* Particularly noteworthy is the observation that eliminating the N-glycosylation site at N141 seems to disrupt the assembly and possibly the function of the AChR. The profound effects of replacing any of the four extracellular cysteines by a serine are



Two schemes of the structure of the alpha subunit of the acetylcholine receptor in a membrane. On the top left it is shown forming an ion channel. The larger scheme illustrates the disposition of parts of the protein chain relative to the membrane, and the positions and effects of a variety of deliberate amino-acid substitutions or deletions. Labelling is explained in the text.

also particularly striking.

Now what must be done? The analysis will have to be extended to provide a more complete explication of structure/function relations. In particular, the examination of effects will have to go beyond the mostly 'yes-no' approach of this initial investigation and define, at the level of single channel recording, the effects of the various modifications and their implications. Detailed biophysical studies will be required to discover the changes in binding,

gating, permeation and selectivity that result from the modifications, and to account quantitatively for these effects in terms of molecular events. Much remains to be done before we have a complete molecular explanation for electrical excitability, but the Numa laboratory has made an exciting start and pointed the way for future investigations. □

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Immunology

Lymphokines and interleukins emerge from the primeval soup

from Marc Feldmann

AT THE the fourth Lymphokine Workshop, held at Schloss Elmau, Germany, on the 17–20 October 1984, reports of the cloning and expression of cDNAs for several lymphokines made it clear that an era of ill-defined research is being replaced by one of elegant cell and molecular biology. The associated prospects for clinical application are having a magnetic attraction for bioengineering and pharmaceutical companies, representatives of which accounted for almost half of the audience.

Over the past 20 years, there have been many reports of potent biological activities in the supernatants of activated lymphoid cells. The term lymphokine was coined to denote those soluble supernatant factors that were not specific for antigen and were thought to be important in, for example, delayed-type hypersensitivity¹. Depending on the bioassay used, many seemingly different lymphokines were described, each

with its own particular acronym but not necessarily with a separate identity. This area of research had a messy, and at times unsavoury, aura for traditional immunologists. Clearly, purification of the molecules was going to be of prime importance to permit correlation of molecular properties with biological function. By 1979, at the second Lymphokine Workshop, there was sufficient confidence in two molecules to call them interleukins — messengers between leucocytes — with interleukin-1 (IL-1) being a macrophage-derived protein involved in T-cell and B-cell activation, and interleukin-2 being the T-cell derived glycoprotein formerly known as T-cell growth factor². Even so, many were dubious at the wisdom of this new classification, doubting whether either IL-1 or IL-2 were single molecules³.

Since the cloning and expression of the first lymphokine gene (that of γ -

interferon⁴) in 1982, several other genes have followed, permitting an analysis of the functions of unambiguous molecules. And all of a sudden, the huge list of reported lymphokines has begun to shrink. For example, the '1a-inducing factor', 'macrophage-activation factor', and a B-cell differentiating factor have all turned out to be the same as γ -interferon. At the workshop, P. Gray and D. Goeddel (Genentech) reported the first cDNA cloning of human lymphotoxin⁷ and tumour necrosis factor⁸, two closely related lymphokines. The former is derived from lymphocytes, the latter from macrophages; both kill tumour cells in experimental systems and so have potential in tumour therapy (see ref.9).

A report of the cDNA cloning of human IL-1, presented by P. Auron (working with C. Dinarello, Tufts University), in the closing stages of the workshop, drew attention to the tension between bioengineering companies and academic researchers. Auron refused to show the IL-1 sequence unless company observers refrained from photographing it. T. Taniguchi (Osaka University) pointed out that in the 2–3 minutes the sequence was discussed, he could have written down 100 bases, more than enough to fish out the cDNA from an appropriate library. The human IL-1 cDNA, of 1,580 bases, was obtained from endotoxin-stimulated monocytes, and encodes a protein of 269 amino acids and relative molecular mass (M_r) 35,000 (35K)¹⁰. The protein is precipitated by Dinarello's antiserum to endogenous pyrogen and precipitation of the pyrogen is inhibited by purified IL-1 of 17K, but the biological activity of the protein is too low to be detected in the usual thymocyte assay. Auron claims that his human IL-1 is similar to the murine IL-1 of Lomedico *et al.*¹¹ which encodes a 33K protein of 270 amino acids. Lomedico *et al.* have shown that the usual 17K IL-1 is the carboxyterminus of the 33K precursor, and that the carboxy-terminal 156-amino acid fragment has very high biological activity. Interestingly, neither human nor murine IL-1 has a classical signal peptide.

Part of the interest in the availability of 'recombinant' IL-1 resides in the need to know whether the multiple effects on different tissues (with some relationship to disease¹²) that have been reported for the substances are due to a single entity or a family of related molecules. The report of a monoclonal antibody to IL-1 (A. Kock and T. Luger, Vienna) may further facilitate biological and biochemical studies of IL-1 activities and the development of new assays.

Once more the question of whether there are macrophage-activating factors apart from γ -interferon was widely discussed. The consensus was that other factors do exist, and M. Lohmann-Matthes (Hannover) reported the separation of a candidate from human γ -interferon in terms of both its chromatofocusing pH and

its activity on mouse macrophages.

The clinical applications of interferon were reviewed by T. Merigan (Stanford University) and R. Oldham (Tennessee). The effectiveness of α - and β -interferons in both severe local and systemic viral infections, especially in immunosuppressed patients, seems established and clinical trials are well advanced. Their effects on cancer are less well established, although Oldham made the point that in phase I and II trials α -interferon has performed much better than the cytotoxic drugs currently in clinical use for cancer. The effects of α -interferon vary dramatically in different cancers, with a reasonable frequency of responders in nodular (non-Hodgkin's) lymphoma, renal cell carcinoma, Kaposi's sarcoma and melanoma. The consensus view was that the clinical effects are due to the antiproliferative effect of interferon rather than its immunoregulatory effects.

Because of the paramount importance of IL-2 in T-cell growth and regulation there was interest in the three reports of the cloning of murine IL-2, by T. Taniguchi¹³, W. Fiers (University of Ghent) and T. Mosmann¹⁴ (DNAX Research Institute). A minor degree of amino acid polymorphism was reported. The presence of twelve glutamines in a row in murine IL-2 may account for the well-known observations that while human IL-2 acts on mouse cells, mouse IL-2 does not act on human cells.

The availability of 'recombinant' human IL-2 in large quantities has allowed it to be tested *in vivo*. M. Lotze (National Institutes of Health) reported that 2 mg of IL-2 (given either as an intravenous bolus or by continuous infusion to compensate for its

short half-life) had profound effects, leading to a transient decrease in T-cells and increase in monocytes, marked and long-lasting eosinophilia and marked gain in weight. These phase I studies precede a prospective trial of IL-2 in cancer patients, based on its beneficial effect on metastatic tumours in mice.

It is evident that, in this field, the impact of molecular biological techniques and bioengineering companies has been profound. In a few years, many of the most interesting mediators have been cloned and expressed in quantities sufficient not only to permit clinical trials, but also to perform definitive biological and pharmacological studies using defined and pure molecules. It is too early to determine whether the clinical prospects of such sledgehammer agents as the interleukins and interferons are very bright, but the cloning of their genes will greatly enhance our knowledge of their function and regulation in both health and disease. $\square$

1. Dumonde, D. *et al.* *Nature* **224**, 38 (1969).
2. Mizel, S. & Farrar, J.J. *Cell Immunol.* **48**, 433 (1979).
3. Larsson, E.-L. *Immun. Today* **3**, 81 (1982).
4. Gray, P. *et al.* *Nature* **295**, 503 (1982).
5. Cohen, S., Pick, E. & Oppenheim, J.J. (eds) *Biology of Lymphokines* (Academic 1979).
6. Taniguchi, T. *et al.* *Nature* **302**, 305 (1983).
7. Gray, P.W. *et al.* *Nature* **312**, 721 (1984).
8. Pennica, D. *et al.* *Nature* **312**, 724 (1984).
9. Sikora, K. *Nature News and Views* **312**, 699 (1984).
10. Auer, P.E. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 7907 (1984).
11. Lomedico, P. *et al.* *Nature* **312**, 458 (1984).
12. Openheim, J.T. & Gery, I. *Immun. Today* **4**, 113 (1982).
13. Kashima, N. *et al.* *Nature* **313**, 402.
14. Yokata, T., *et al.* *Proc. natn. Acad. Sci. U.S.A.* (in the press).

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Immune diseases

Many roles for interleukin-1

from Gordon Duff

ADVANCES in interleukin-1 (IL-1) biology, many of which were discussed at a recent workshop*, raise the prospect of a better understanding of inflammatory and immune-mediated tissue damage, such as occurs in rheumatic diseases. Several cytokines with descriptive names are now thought to be members of the IL-1 group of molecules (see Dinarello, C.A. *Rev. infect. Dis.* **6**, 51; 1984). These include endogenous pyrogen (EP), lymphocyte-activating factor (LAF), leukocytic endogenous mediator (LEM), mononuclear cell factor (MCF), synovial factor (SF), catabolin, proteolysis-inducing factor (PIF) and epidermal cell-derived thymocyte-activating factor (ETAF).

Some confusion has existed over the molecular characteristics of IL-1 poly-

peptides but it is clear that human monocyte IL-1 occurs in two sizes of M_r 15 K and 35 K. Each gives two peaks on chromatofocussing (with pIs of about 5 and 7) making at least four distinct molecular species. Whether these are produced by different genes or from a single gene product processed in different ways is not known yet. Larger molecules, of greater than 50 K with some IL-1 activities, may either be polymers of the 15 K molecule or complexes of this molecule with carrier protein (P. Murphey, Johns Hopkins); smaller molecules (2 K, 4 K) found in human urine and with thymocyte-activating properties but no pyrogenic activity are probably fragments of 15 K IL-1 lacking the full complement of active sites (E. Kimball, Ayerst Laboratories). Pig IL-1 (catabolin) has a pI of 5 and seems antigenically related to the acidic form of human IL-1 (J. Saklatvala, Strangeways, Cambridge). C.A. Dinarello (Tufts)

reported the use of IL-1 cDNA clones as probes to characterize and isolate human monocyte IL-1 mRNA which, after translation, gave a 35 K polypeptide thought to be a precursor of biologically active IL-1. It seems that the duration and type of stimulus may influence the expression of mRNA for different MCF/IL-1 activities, some of which remain in the cell while others are secreted (J.-M. Dayer, University of Geneva). J. van Damme (University of Leuven, Ghent) described part of the sequence of a peptide that stimulates β -interferon production and also has several IL-1 activities, suggesting that interferon induction may be yet another property of the IL-1 family.

Mononuclear phagocytes are considered the predominant source of IL-1 but it is clear that it (or a similar mediator) can also be produced by other cell types, including epidermal cells (the source of ETAF), renal mesangial cells, astrocytes, skin Langerhan cells and, in some circumstances, B-cells. At the workshop, it was reported that IL-1 is present in brain cells of rats with experimental allergic encephalitis and of mice treated with intraperitoneal lipopolysaccharide (A. Fontana, University of Zurich). Dendritic cells from synovial tissue of patients with rheumatoid arthritis also produce IL-1. Direct stimuli for its production include many microbial products or their analogues. Vitamin D₃ and lymphokines, such as γ -interferon, can augment this process and hypoxia may stimulate IL-1-producing cells in the lung. The use of cell lines such as macrophage-related U937 (human), P388D1 (murine) and human squamous cell carcinoma should be helpful in determining the factors governing IL-1 production, and establishing the relevance of reports that different stimuli result in the appearance of biologically-distinct forms of IL-1.

In connective tissue cells, catabolin and MCF seem mostly to promote catabolism of structural protein and matrix by stimulation of proteolytic enzymes and prostaglandins, but with concomitant reduction of collagen and proteoglycan synthesis (Saklatvala; R.G. Russell, University of Sheffield). Fibroblasts produce prostaglandin E in response to IL-1, and though IL-1 may promote fibroblast proliferation, other macrophage-derived growth factors probably contribute to this (S.J. Leibovich, North Western University Chicago). It was reported that, in response to IL-1, bone osteoblasts proliferate and generate a short-range signal for osteoclast activation. While IL-1 seems to be distinct from osteoclast activating factor (a lymphokine), a circulating 4K inducer of muscle proteolysis (PIF) is probably a stable fragment of IL-1.

In the immune system, the relations between antigen presentation, the expression of class II major histocompatibility antigens and IL-1, led S.K. Durum (NCI, Frederick) and Y. Ron (Scripps Institute, La Jolla) to suggest that surface IA

*The First 'International Workshop on Interleukin-1', Grays, Essex, 24-25 November 1984 was organized by the British Society for Rheumatology. The proceedings will be in the May 1985 supplement to the British Journal of Rheumatology.

molecules participate in the pathway leading to IL-1 release—possibly by acting as receptors for activating signals. E. Atkins (Yale), who previously described lymphocyte-mediated IL-1 production in delayed-type hypersensitivity, provided evidence of cell-mediated suppression of this IL-1 release in desensitized animals. That IL-1 itself may have a major effect on the balance between positive immunity and suppression was raised by D. Green (Yale).

As a stimulator of the acute phase response, it seems that brain-mediated, as well as direct hepatic effects, of IL-1 may be important. Patients with rheumatic diseases show varying acute phase responses to different stimuli and disease activity may influence IL-1 responsiveness. In active rheumatoid arthritis, the high levels of synovial fluid IL-1, the characteristic features of cartilage destruction, synovial proliferation, adjacent osteopenia and muscle wasting, and the systemic changes of acute phase response, lymphocyte activation and fever, suggest that over-production of IL-1 might be a key pathogenic factor. In both rheumatoid arthritis and adjuvant arthritis there is also disordered IL-2 production and/or responsiveness. Since crystals such as monosodium urate induce the

production of IL-1, the production of IL-1 is also likely to contribute to the inflammatory response in diseases such as gout. On the positive side, pretreatment of immunocompromized (protein-starved) guinea pigs with IL-1 leads to a marked increase in host defence against gram-negative bacteria (L. Moldawer, Harvard University, Boston).

That IL-1 may have a role in normal physiology is indicated by the finding that it can be detected in the plasma of healthy women and that levels vary with the menstrual cycle (J. Cannon, Tufts University Boston). So IL-1 appears to be a family of related peptides with local and distant actions on many different tissues. Dysregulation of IL-1 production and its effects may contribute to the pathology of several types of disease. The analogy to a hormone serving host defence and the maintenance of tissue integrity is attractive but, as yet, speculative. With the DNA cloning of some IL-1 activities and the prospect of 'recombinant' IL-1s, this field holds much future promise for both biologists and clinicians. □

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Genetic engineering

Eukaryotic proteins retargetted among cell compartments

from John Ellis

UNLIKE bacteria, the cells of plants and animals are subdivided into many membrane-enclosed compartments, such as the nucleus, mitochondria and chloroplasts. Each compartment contains its own characteristic complement of proteins which enables that compartment to carry out its metabolic roles. With the exception of a small number of proteins in chloroplasts and mitochondria, all the proteins of compartments are synthesized on the ribosomes of the cell cytosol, and so have to be transported across one or more membranes to their specific destination. It follows that there must be targetting information within each protein. One way to uncover this information is to follow the intracellular fate of chimaeric proteins composed of parts of two proteins that reside in different compartments. On page 358 of this issue Van den Broeck *et al.* report the first use of this approach to insert a foreign protein into chloroplasts, while a spate of recent papers describes the insertion of foreign proteins into other compartments—the nucleus, the mitochondrion and the endoplasmic reticulum. The ability to retarget proteins into subcellular compartments is an exciting development since it opens up a large range of possibilities for the genetic engineer.

Most transported proteins are synthesized with an extra sequence of amino acid residues at their amino terminus; this extension, or prepiece, is essential for correct targetting, and is removed enzymatically once transport into the target compartment has been initiated^{2,3}. In leaves of the pea plant (*Pisum sativum*) the small subunit of the CO₂-fixing enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase, is synthesized by cytosolic ribosomes as a precursor with a 57 amino-acid prepiece; after its release from the ribosome, the precursor form is transported across the chloroplast envelope into the stromal compartment. How this transport takes place is not known, but it is prevented if the prepiece is removed. But does the prepiece contain all the information that is necessary to target the enzyme to the chloroplast? To answer that question, Van den Broeck *et al.* have fused the DNA coding for the prepiece to the structural gene for neomycin phosphotransferase, an enzyme of the cytosol of bacterial cells that confers resistance to antibiotics, such as neomycin and kanamycin, by adding phosphate groups to them. With the aid of the *Agrobacterium* transformation system, the chimaeric gene was inserted into the cells of tobacco stems. The resulting transformed cells yielded

calli that grow in the presence of kanamycin and contain neomycin phosphotransferase. The critical observation is that this enzyme is present in soluble form inside intact chloroplasts isolated from the calli; however, when the DNA sequence encoding the prepiece is omitted from the chimaeric gene, the enzyme is found in the cytosolic fraction of the calli. In other words, the addition of a prepiece from a protein that normally enters chloroplasts is sufficient to cause a cytosolic protein to enter the chloroplast compartment.

Similar experiments using chimaeric genes inserted into animal and yeast cells have shown that it is possible to retarget proteins into three other compartments: the nucleus, the mitochondrion and the endoplasmic reticulum. The underlying assumption of all these experiments is that locational information resides within discrete amino-acid sequences that occur either within the prepiece or, in some cases, within the mature protein. A further assumption is that these sequences possess a degree of autonomy, so that sequences on either side of them are not critical for their targetting function.

A wide range of nuclear proteins will migrate into the nucleus when micro-injected into the cytoplasm of animal cells, suggesting that these mature proteins contain locational information⁴. Fusion of the amino-terminal sequence of a presumptive nuclear protein of yeast to the bacterial enzyme β -galactosidase results in the accumulation of the enzyme in the nuclei of yeast cells⁵. In cultured monkey cells infected with the virus SV40, the viral protein, large-T antigen, accumulates in the nucleus as a result of a sequence of about seven amino acids that includes a critical lysine residue at position 128. Attachment of regions containing this sequence to the amino termini of the cytosolic enzymes β -galactosidase and pyruvate kinase is sufficient to cause them to accumulate in the nucleus, as judged by immunofluorescence⁶. Replacement of lysine 128 by threonine is sufficient to prevent their nuclear accumulation. Similarly, a fusion protein consisting of a specific portion of the influenza virus nucleoprotein joined to globin has been shown to accumulate in the nuclei of *Xenopus* oocytes⁷. In this experiment, the nuclear and cytosolic compartments were physically separated so that pulse-chase experiments could be carried out, to rule out the possibility that the observed distribution of the chimaeric proteins is due to preferential degradation in one of the compartments. In addition, manual removal of the nuclear envelope has definitively localized the chimaeric protein within the nucleoplasm (A. Colman, personal communication).

Two cytosolic enzymes have been retargetted to yeast mitochondria by the addition to their amino termini of sequences derived from imported mitochondrial proteins. In one case, the attachment of the 350 amino-terminal amino acid residues of the

β subunit of mitochondrial F_1 -ATPase to a large enzymatically-active fragment of bacterial β -galactosidase results in the presence of β -galactosidase in the mitochondria of yeast cells transformed with the chimeric gene; the enzyme activity is resistant to added protease⁸. In a similar approach, the addition of 61 amino-terminal residues of a protein of the outer mitochondrial membrane to the amino terminus of the same β -galactosidase fragment has been shown to be sufficient to bind the β -galactosidase to that membrane⁹; in this case the enzyme is susceptible to added protease. The most impressive result is the transport of the mouse cytosolic enzyme dihydrofolate reductase into isolated yeast mitochondria when 22 of the 25 amino acid residues of the prepiece of cytochrome oxidase subunit IV are fused to the amino terminus of the reductase¹⁰. The fusion proteins transported across both mitochondrial membranes in the matrix space. In more recent experiments it has been shown that the first 15 amino acid residues of the prepiece are sufficient for retargeting (G. Schatz, personal communication). All these experiments indicate that the carboxy-terminal sequences of imported mitochondrial proteins are not required for correct targeting; some portion of the amino-terminal sequence is all that is required.

Other experiments have shown that it is possible to redirect globin from the cytoplasm into the endoplasmic reticulum, both *in vivo*¹¹ and *in vitro*¹², by fusing it with appropriate sequences from secreted

proteins. Of particular interest is the identification of the locational signal in the secreted protein ovalbumin, which has no prepiece, as residing in amino acid residues 22–41; attachment of this sequence to the amino terminus of chimpanzee α -globin results in the sequestering of the fused protein in the endoplasmic reticulum of *Xenopus* oocytes¹¹.

Future work will determine how far these exciting observations can be extended to other locational sequences and to other cytosolic proteins. This ability to retarget proteins will not only stimulate more incisive experiments on the mechanism of protein transport, but also will allow the directed genetic modification of metabolic pathways in the subcellular compartments of eukaryotic cells. □

1. Van den Broeck, G., *et al.* *Nature* **313**, 358 (1985).
2. Silhavy, T.J., Benson, S.A. & Emr, S.D. *Microbiol. Rev.* **47**, 313 (1983).
3. Carne, T. & Scheele, G. in *Cell Biology of the Secretory Process*, 73 (Karger, Basel, 1984).
4. De Robertis, E.M. *Cell* **32**, 1021 (1983).
5. Hall, M.N., Hereford, L. & Herskowitz, I. *Cell* **36**, 1057 (1984).
6. Kalderon, D., Roberts, B.L., Richardson, W.D. & Smith, A.E. *Cell* **39**, 499 (1984).
7. Davey, J., Dimmock, N.J. & Colman, A. *Cell* (in the press).
8. Douglas, M.G., Geller, B.L. & Emr, S.D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3983 (1984).
9. Hase, T., Müller, U., Riezman, H. & Schatz, G. *EMBO J.* **3**, 3157 (1984).
10. Hurt, E.C., Pesold-Hurt, B. & Schatz, G. *FEBS Lett.* **178**, 306 (1984).
11. Tabe, L., *et al.* *J. molec. Biol.* (in the press).
12. Lingappa, V.R., Chadez, J., Yost, C.S. & Hedgepeth, J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 456 (1984).

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Atmospheric chemistry

US plans for increased research

from S.A. Penkett

TOGETHER with a greater understanding of chemical phenomena in the lower atmosphere, the past decade has brought predictions of a series of potential anthropogenic catastrophes, such as the greenhouse effect and a nuclear winter. To try and rationalize this situation and to provide a solid basis of fact, a panel of the US National Academy of Sciences (NAS), chaired by R.A. Duce, has recently advocated a large international effort in basic atmospheric science, concentrating on the troposphere. The programme will be led by the US National Science Foundation (NSF), National Aeronautic and Space Administration and National Oceanic and Atmospheric Administration. The proposals are published in a report entitled *Global Tropospheric Chemistry: A Plan of Action* (National Academy, Washington D.C. 1984). As well as outlining the need for a programme and proposing specific large-scale experiments in the atmosphere, the report presents an almost encyclopaedic synopsis of existing knowledge, sufficient to form the basis for an advanced university course in atmospheric chemistry.

Tropospheric chemistry deals with the chemical cycling of many nutrient elements that are essential for biological activity. It therefore becomes a study of the pathways of many minor constituents through the atmosphere and draws its format from other earth sciences, such as geochemistry and hydrology. A typical cycle is made up of many individual processes. It begins when gases in various chemical forms are emitted from the biosphere, either over land masses or over the ocean, and there is a need to quantify in detail the emissions from various biomes, such as tropical forests, agricultural areas and oceans. The emitted gases are spread by air movements and the resulting composition of the atmosphere will vary greatly according to the location of the source area and the chemical reactivity of the compound in question. Thus large-scale experiments are required to study the global variability in atmospheric compositions. For now, this is probably best done by aircraft and ships, but remote sensing from satellites of certain key molecules, such as CO, H₂O vapour and O₃, is highly desirable.

The many trace gas species undergo chemical transformations which are driven by the photochemical production of free radicals and peroxides in the troposphere. For example, in the case of carbon, CH₄ is oxidized to CH₃O in the gas phase by hydroxyl radicals. This can then be photolysed to CO, which is ultimately converted to CO₂, again by reaction with hydroxyl radicals. Other atmospheric reactions include the conversion of SO₂ to H₂SO₄, this time by reaction with soluble peroxides in cloud droplets. Comprehensive tests of the photochemical theory, which attempts to explain the presence of free radicals and peroxides in the troposphere, are essential.

The molecular products of the oxidation processes tend to be more easily removed from the atmosphere, either by rain or by gaseous absorption at the Earth's surface. The report calls for large-scale experiments to study the removal processes in detail, including budgetary experiments on cyclonic storm systems for wet removal and flux experiments on many gases for dry removal. Particular studies will investigate the nature of transfer processes which occur at interfaces such as the ocean surface and the tropopause.

In addition to the need for field and laboratory experiments, the report draws attention to the need to develop numerical models of increasing insight and complexity. This will probably need little encouragement, to judge by the plethora of theories and predictions that have already been based on a very limited data set.

There is no doubt that a large programme of research into the fundamental processes of atmospheric chemistry is needed; in many cases, the analytical capability is already to hand and the observation platforms are available. There is also no doubt that the programme needs to be international. Atmospheric gases are widespread across the world. A positive response has already been made to NSF and NAS by the UK Natural Environment Research Council, and others are expected. Whether, in the present financial climate, the necessary funds will be made available for this quiet revolution in atmospheric science remains to be seen. Meanwhile, a panel, chaired by R. Cicerone and W. Prinn, and consisting of US and non-US atmospheric scientists, has been set up to prepare estimates of cost and feasibility for a variety of programmes. This, it is hoped, will provide a specific stimulus for the funding agencies. □

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Corrigendum

IN the obituary of Milan Hašek (*Nature* **313**, 96; 10 January 1985) by P.B. Medawar and L. Brent, "negative hybridization" should have read "vegetative hybridization".

A comparison of terrestrial and marine ecological systems

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I review here the differences between temporal variability in terrestrial and marine environments and consider how this external forcing may affect population fluctuations in the two systems. The internal dynamics and community responses are expected to differ significantly with marine populations more likely to show longer term changes between alternative community structures.

THREE essential features are used for conceptual or numerical models of natural systems: (1) a high order of nonlinearity, (2) large variability in the forcing functions and (3) a wide range of space and time scales. It is impossible to combine all three in any single model, so major simplifications are introduced. I discuss here the effects of variable frequency of forcing, regarding atmospheric and oceanic physical variations as the input and corresponding population changes as output. Two simple models reproduce some, but not all, of these input/output relations and may contribute to our understanding of the differences in response of marine and terrestrial ecological systems to these parameters.

Physical systems

The separation of 'noise' from 'signal' in terms of temporal variation corresponds to the distinction between predictable and unpredictable fluctuations in the physical environment. If regular diurnal, lunar and seasonal cycles are removed, there is a large residual variability which can be considered as inherently unpredictable in a strictly deterministic sense. White noise, defined as constant variance per unit frequency, is the statistically simplest and theoretically preferred form to describe this variability¹. (The total variance of white noise defined in this way is unbounded as frequency (f) tends to infinity. One must assume a cut-off at high frequencies which corresponds technically to instrument response time; conceptually it is assumed that the world is predictable on very short time scales.) Is white noise an appropriate representation of variability, particularly on the longer inter-annual or decadal time scales?

For terrestrial environments, temperature records and other data analysed in terms of their variance spectra² show (Fig. 1a) that white noise is an adequate representation down to frequencies of $\sim 0.02 \text{ yr}^{-1}$. For time scales longer than 50 yr, the variance increases significantly and the spectrum becomes 'red' (by analogy with the spectrum of red light), but the use of white noise can be appropriate for simulation of the random forcing of terrestrial ecosystems up to several decades.

The marine environment is very different. Long-term temperature records from the deep ocean³ show a spectrum where the variance increases continuously from hours to years with, approximately, variance (v)/unit frequency proportional to f^{-2} . Records of sea-level changes have been maintained for longer periods⁴ and are an index of general changes in temperature, ocean circulation and density structure⁵. At much longer (geological) time scales, there are general relations between temperature and sea level⁶⁻⁸ so that the spectrum from geological data can be plotted together with the instrumental measurements (Fig. 1b). There is a gap at 10–1,000-yr, but interpolation would give a spectrum that decreases continuously with f .

Should one expect marine spectra to be red over the entire time range from days to millennia? A simple stochastic model of the forcing of an ocean by atmospheric white noise⁹ shows that the much longer time scales of heat transport and transfer

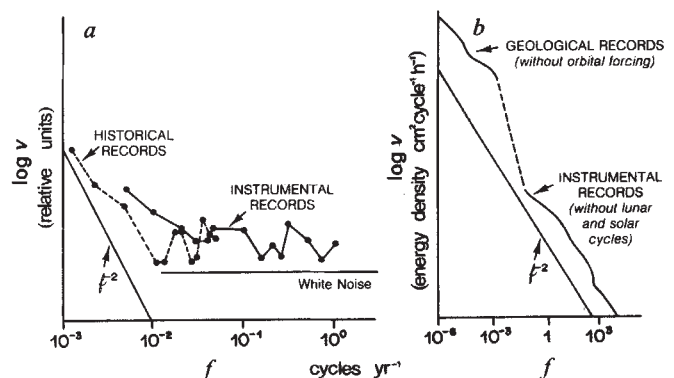


Fig. 1 Frequency spectra of variance for a, atmospheric temperature in England (redrawn from US National Academy of Science Report²) and b, sea level combining instrumental⁴ and geological⁶ data (J. Imbrie, personal communication).

in the ocean, $\leq 1,000 \text{ yr}$, can produce a red spectrum in the ocean's response so that $v \propto f^{-2}$ is an appropriate index for those external factors perturbing marine ecosystems. Again, there are technical and conceptual problems for the integrated variance over the whole frequency range. The total variance is bounded as f tends to infinity, so that the 'terrestrial' problem does not arise and the marine world is predictable at small time scales. But as f tends to zero, the total variance is unbounded, which no ecological system could assimilate. Thus, at very long time scales the system is inherently unpredictable and must be considered in the evolutionary, rather than the ecological, context; this brings into consideration questions such as 'punctuated equilibria'¹⁰. It is of interest also to ask whether the appearance of red noise in the atmospheric records at 50–100 yr is a reflection of the close coupling of the air and sea systems at and beyond these scales.

Ecological systems

Examples of relatively long-term population changes in terrestrial systems¹¹ show the variety of patterns involving persistence, cycles and trends. It was concluded that 'nearly all natural populations are characterized by patterns of change that keep their numbers within bounds... only a minority of populations fluctuate so wildly that an equilibrium level is not obvious' (ref. 11).

Where very large changes do occur at fairly regular intervals (Fig. 2), it is assumed normally that internal features of the ecosystem dynamics are responsible. For example, the spruce budworm cycle of tree growth and defoliation¹² is assumed to have two equilibrium states, where the 37-yr-cycle is determined by tree growth rates and the rapid jump between states depends on the fast reproductive rate of the budworm. Climate variation does not seem to be significant.

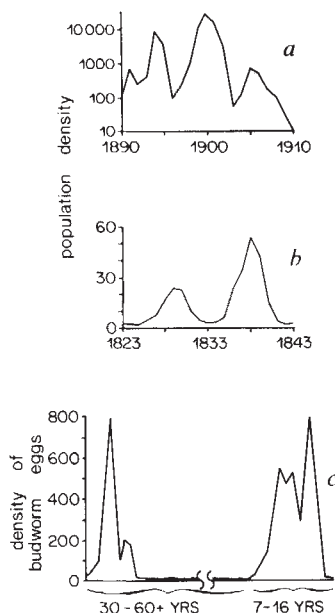


Fig. 2 Population changes in terrestrial species that show evidence of cyclical response: *a*, moth, *Bupalus*¹¹; *b*, Arctic lynx¹¹; *c*, spruce budworm¹².

Long-term information about marine ecosystems is dependent mainly on data from pelagic fish populations¹³. The longest records, covering 2,000 yr, are from fish scales in sediments off southern California¹⁴. A major feature is the relatively regular appearance and disappearance of sardines about every 70 yr, but these sediment records also show the continuous presence of anchovy and hake. From commercial fisheries there is historical evidence for the appearance and disappearance of breeding stocks of species such as herring (Fig. 3), mackerel and pilchard¹⁵ over relatively large regions. In recent years, heavy fishing has altered the magnitude of population changes but maintained the rough periodicity of 50–100 yr¹⁶. In one case¹⁵ where long-term data are available on community structure, many other species also changed whereas essential nutrients remained nearly constant (Fig. 3).

Historically, therefore, many pelagic stocks display marked fluctuations between states of high and low abundance. These alternative abundance levels may be regarded as two different equilibrium conditions, but the jumps between the two states are not considered in terms of the internal dynamics. They are connected usually to longer-term climate trends¹, although there are no simple relations between the physical and biological changes.

Theoretical models

To illustrate the relation between internal, biological, rates of change and external forcing at different frequencies, consider the response of a simple system with two equilibrium states $x = \pm 1$ subject to perturbation at a range of frequencies¹⁷ (analysis provided by Dr Donald Ludwig).

$$\frac{1}{r} \frac{dx}{dt} = \begin{cases} -x + 1 \\ -x - 1 \end{cases}$$

where r is the response rate and the waiting time between switches has an exponential distribution with mean q^{-1} .

If $q \ll r$, the probability is overwhelming that x will be near +1 or -1. Thus the stationary distribution is peaked at both ends of this range (Fig. 4a). If $q \gg r$, the two dynamics are united and effectively $dx/dt = -rx$, where the most probable state (Fig. 4b) is between the equilibrium positions. If $q = r$, the distribution is uniform and the time sequence would be roughly and irregularly cyclical. More generally, if $q = 0(r)$, then the solution depends on the exact form of the system rather than only on the existence of two equilibrium conditions (Fig. 4c).

This model describes the essential response of systems with two equilibria to particular frequencies of stochastic variation. But the external environment contains random fluctuations at such a wide range of frequencies that both responses (Fig. 5a, b) may be expected to occur. Thus the quantitative distribution of the variance is highly significant. If we assume that the effects for terrestrial and marine systems will depend on the character of the physical frequency distributions, then the general qualitative response of these systems could be inherently different.

For marine systems with the ecological effects of variability proportional to the physical relation derived earlier, one would expect the system to be dominated by the lower frequencies. In consequence, the expected distribution (Fig. 4a) is similar to that derived from some of the long-term pelagic fish stock records. For terrestrial systems, equal weight would be given by white noise to the very different responses in Figs. 4a, b, so no conclusions would emerge about the character of the expected distributions. The output would depend on the internal dynamics of the system and the system could display persistence, cycles or occasional abrupt change. The evolution of system structure would tend to lead to the first category¹¹.

This very simple model has the advantages of analytical tractability and apparent generality. More complicated models contain specific descriptions of ecological relations and usually require numerical solution, particularly with stochastic inputs. One such model for a population (P) will be used here to illustrate the possible responses when red or white noise is applied over a range of frequencies¹⁸.

$$dP/dt = P(1 - P/b) - cP^2/(1 + P^2)$$

combines a logistic growth with sigmoidal-shaped predation;

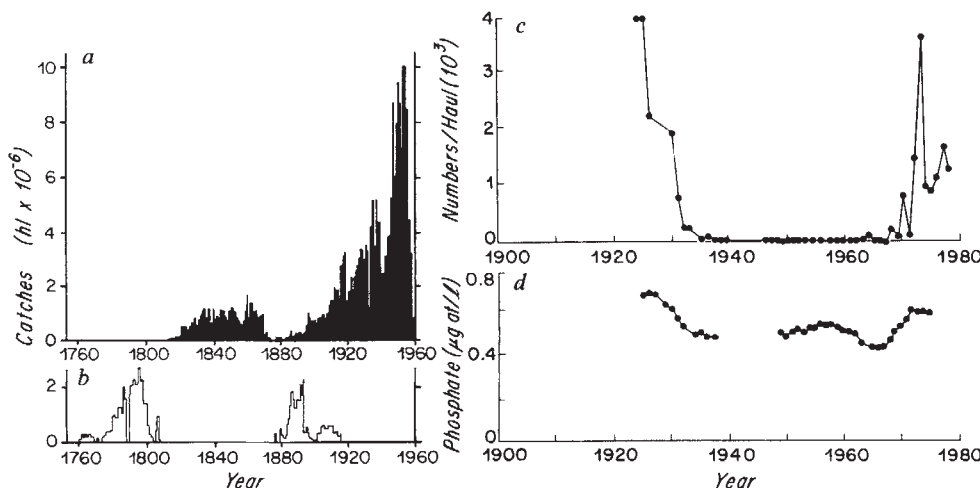


Fig. 3 Changes in marine species: *a*, Norwegian herring fishery¹⁶; *b*, Swedish herring fishery¹⁶; *c*, *Sagitta elegans* in English Channel¹⁵; *d*, winter phosphate concentration in English Channel¹⁵.

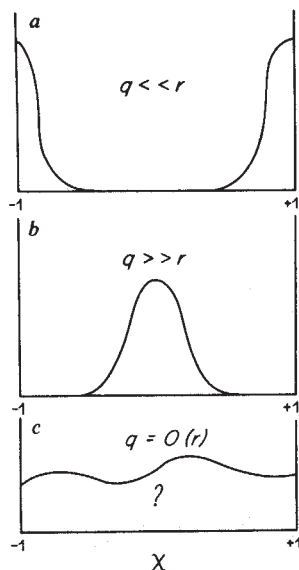


Fig. 4 Response of a two-equilibria system to stochastic forcing at frequencies: *a*, much less than and *b*, much greater than the intrinsic response rate of the system. *c*, Indeterminacy of the system when the rates are comparable.

b is carrying capacity; *c* is mortality rate. The general applicability has been reviewed¹⁹ and used in specific applications for the spruce budworm¹² and for marine plankton²⁰. For appropriate values of *b* and *c* the system has two equilibrium states; by varying *c* stochastically across these states, time sequences in the response of *P* are generated for different frequency ranges of red and white noise¹⁸.

For red noise applied to the coefficient over a frequency range *f* to 30 *f*, the system changes from a single to a double equilibrium condition at a value of *f* = 0.015 (in arbitrary units; Fig. 5*a*). For white noise with the same total variance in *c* and the same value of *b* (= 10), the system does not begin to depart significantly from a single equilibrium state until *f* = 0.003 and then the response is highly irregular (Fig. 5*b*). For a time scale in years then, with red noise, the system would jump regularly when the longest period reached 70 yr. With white noise, however, the comparable forcing period is ~300 yr, but the system would then fluctuate irregularly at much shorter time scales. Thus, with the same model there are very different characteristic responses when the system is subject to external forcing by white and red noise typical of terrestrial and marine environments. Do physical fluctuations have a direct effect on the two types of systems in proportion to their physical amplitudes? Or do the mechanisms within the system accept, modify or eliminate the effects of this variability?

Discussion

Should comparisons of ecosystems include both terrestrial and open-sea examples? There is considerable interest and controversy about general ideas, such as resilience²¹, which presuppose the concept of multiple equilibrium states with stochastic forcing. In one example, the concept of multiple equilibria is rejected but very limiting criteria are imposed: "We will deal only with the question of whether, under the same climate regime, populations are stable or persistent in the face of discrete, punctuated disturbances"²². Such criteria are not merely restrictive, but eliminate major features of both domains and, especially, the differences between them.

The inherent 'climate' of the ocean is unlike that of the land where the assumption of constant variance is acceptable up to periods of ~50 yr. In the sea, short-term variability is damped out by the very large heat capacity of the ocean. But in turn this large thermal capacity and the long period exchange rates between deep and near-surface waters (in the order of 10²–10³ yr) leads to relatively large-amplitude changes at long time

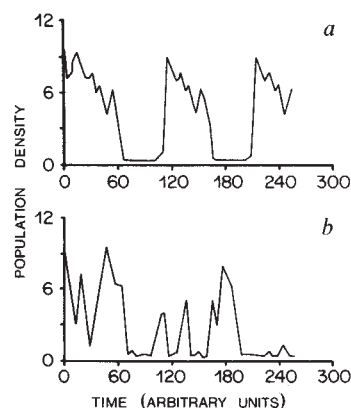


Fig. 5 Response of *P* to stochastic forcing by red noise (*a*) and white noise (*b*) (see text for details).

scales. Simple models can demonstrate the very different responses to these alternative forms of variability and some of the features of the two systems might be explicable in terms of differences in forcing by external physical factors. On the much longer (evolutionary) time scales, the terrestrial and marine ecosystems could develop and adapt differently in relation to their particular environmental structure.

The dominance of poikilotherms in the marine environment even at higher trophic levels is an obvious consequence not only of the much lower absolute range in diurnal and seasonal cycles, but of the smaller variability at short and medium time scales. There is little need (or opportunity) for individual pelagic organisms to create their own internal or immediate environment, unlike their terrestrial counterparts (including, in evolutionary terms, the marine mammals). At the population level, successful reproduction depends on a combination of physical dispersal and relatively predictable food cycles. Energetically and behaviourally, this is a preferable solution to close supervision and feeding of the young. From fish recruitment data there is little if any relation between stock size and recruitment on a year-to-year basis and the early life processes are decoupled from the adult state. This strategy is very different from that of most terrestrial populations, particularly vertebrates.

These differences in strategies make it difficult to relate concepts derived from terrestrial ecology to marine populations. For fish populations, the decoupling of larval and adult phases with their very different feeding and mortality patterns makes the *r-k* concepts inapplicable, even though the logistic relation may still be a useful simplification. Similar mathematical expressions for population growth or mortality may be derived from quite different ecological assumptions.

A simple definition of an ecosystem¹ is of an isolated community, uniformly unvarying in space, with time the only dependent variable. This definition is difficult to apply on land and even more difficult in the sea. The assumptions in this definition allow average food web and energy flow diagrams²³, but they fail completely to explain the switch²⁴ from one structure ('herring') to another ('haddock').

The two environments also have different spatial characters. Physical dispersion in the sea can blur the boundaries between regional systems and recruitment failures in one region may be reversed in suitable conditions by passive input from neighbouring breeding stocks. Similar processes on land usually require active migration between discrete locations. Further, the patchiness in marine organisms, combined with dispersion, may provide stabilizing mechanisms²⁵ mathematically similar to the functional responses in terrestrial animals^{26,27}, but with a different behavioural and energetic basis.

A 'terrestrial' system where environmental variability is large at both short- and long-term periods could be expected to

develop mechanisms internal to the system which would cope with short-term variability and in so doing, would minimize also the effects of longer-term variations. Such assumptions about internal mechanisms combined with relative immobility lead easily to the ecosystem concept with the emphasis, mathematically, on regulatory formulations independent of external variability. In 'marine' systems, less robust internal processes are needed to handle the smaller amplitude variability at short periods commensurate with the life span of the organisms. The possible absence of such mechanisms, combined with increasing variance with period, can mean that marine populations or ecosystems not only have different ways of dealing with short-term variance but also will respond differently at longer time scales. The dominant processes of numerically large larval reproduction, combined with extensive potential dispersal, means that replacement species may be available always. Thus deterministic ecosystem concepts and models are not so easily applicable.

On this basis, one should expect the internal dynamics and structures of marine and terrestrial systems, particularly at the

higher trophic levels, to differ in significant ways in response to the temporal character of each physical environment. This does not mean that comparisons cannot and should not be made. Populations and communities face the same general requirements of long-term survival and evolution in the context of unpredictable environments. The concept of persistence within stochastically-defined bounds²² demands explicit attention to the time (and space) characteristics of the variability. The fundamental differences in the two 'climates' should lead to significantly different types of system and so provide better tests of general hypotheses about resilience, food web dynamics and other ecological concepts.

I thank Don Ludwig who suggested the first model used here and its ecological relevance, John Imbrie and Nicholas Shackleton for providing their unpublished construction of sea-level variability used in Fig. 2, Eric Henderson for simulations for the second model, with support from the Coastal Research Center, and the Center for the Analysis of Marine Systems of WHOI. Contribution 5795 from the Woods Hole Oceanographic Institution.

1. May, R. M. *Stability and Complexity in Model Ecosystems* 2nd edn (Princeton University Press, 1974).
2. U.S. Committee for the Global Atmospheric Research Program *Understanding Climatic Change: A Program for Action* (National Academy Press, Washington, 1975).
3. Wunsch, C. *Evolution of Physical Oceanography* (eds Warren, B. A. & Wunsch, C.) 342-374 (MIT, Cambridge, 1981).
4. Munk, W. H. & Cartwright, D. E. *Phil. Trans. R. Soc.* **259**, 533-581 (1966).
5. Chelton, D. B., Bernal, P. A. & McGowan, J. A. *J. mar. Res.* **40**, 1095-1125 (1982).
6. Shackleton, N. J. & Opdyke, N. D. *Quat. Res.* **3**, 39-55 (1973).
7. Fairbanks, R. G. & Matthews, R. K. *Quat. Res.* **10**, 181-196 (1978).
8. Hays, J. D., Imbrie, J. & Shackleton, N. J. *Science* **194**, 1121-1132 (1976).
9. Lemke, P. *Tellus* **29**, 385-392 (1977).
10. Gould, S. J. & Eldredge, N. *Paleobiology* **3**, 115-151 (1971).
11. Pimm, S. *Food Webs* (Chapman & Hall, London, 1982).
12. Ludwig, D., Jones, D. D. & Holling, C. S. *J. anim. Ecol.* **47**, 315-332 (1978).

13. Cushing, D. H. *Climate and Fisheries* (Academic, London, 1982).
14. Soutar, A. & Isaacs, J. D. *Fishery Bull. Fish Wildl. Serv. U.S.* **72**, 257-275 (1974).
15. Southward, A. J. *Nature* **285**, 361-366 (1980).
16. Devold, F. *Rapp. P.-v. Réun. Cons. perm. int. Explor. Mer.* **154**, 98-108 (1963).
17. Slatkin, M. *Ecology* **59**, 249-256 (1978).
18. Steele, J. H. & Henderson, E. W. *Science* **224**, 985-987 (1984).
19. May, R. M. *Nature* **269**, 471-477 (1977).
20. Steele, J. H. & Henderson, E. W. *Am. Nat.* **117**, 676-691 (1981).
21. Holling, C. S. *A. Rev. Ecol. Syst.* **4**, 1-23 (1973).
22. Connell, J. H. & Sousa, W. P. *Am. Nat.* **121**, 789-824 (1983).
23. Steele, J. H. *The Structure of Marine Ecosystems* (Harvard University Press, 1974).
24. Steele, J. H. *Applied Biology* **4**, 103-140 (1979).
25. Steele, J. H. *Oceanus* **23**, 3-8 (1980).
26. Holling, C. S. *Mem. ent. Soc. Can.* **45**, 1-60 (1965).
27. Hassell, M. P., Lawton, J. H. & Beddington, J. R. *J. anim. Ecol.* **46**, 249-262 (1977).

ARTICLES

Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase

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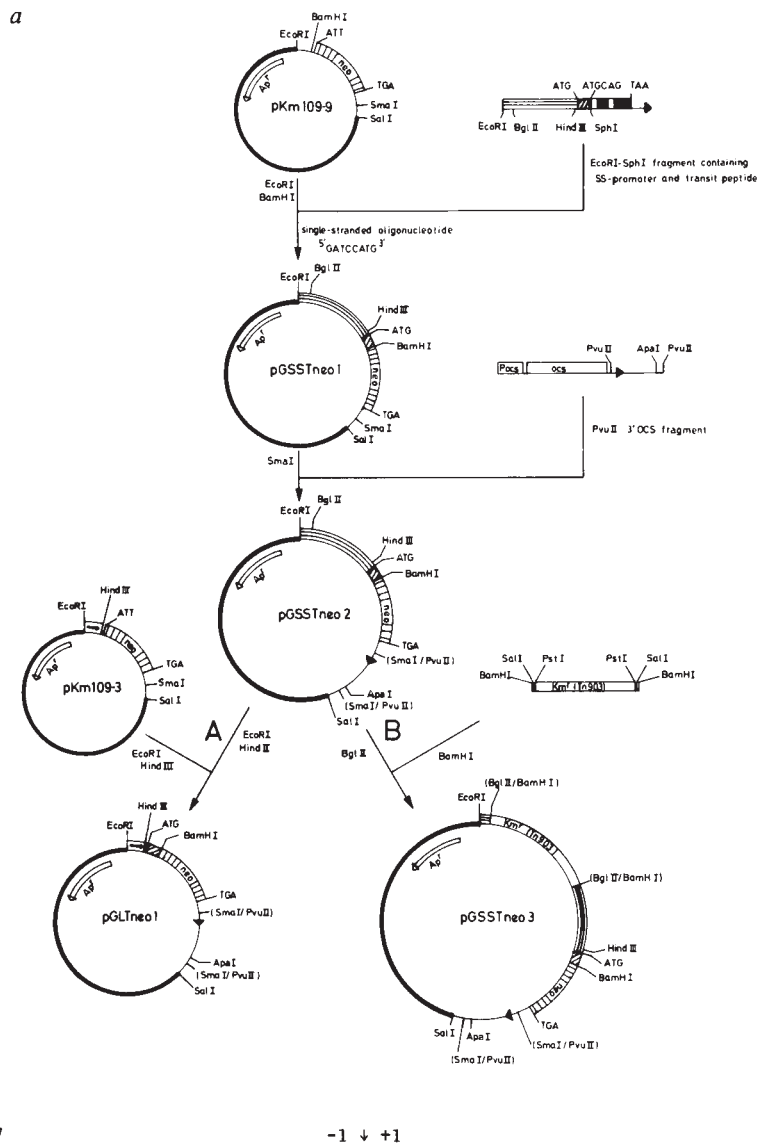
Chimaeric genes can be constructed which fuse the transit peptide of a small subunit of the chloroplast-located ribulose 1,5-bisphosphate carboxylase with a bacterial protein. The fusion protein is translocated into chloroplasts and cleaved in a similar way to the small subunit polypeptide precursor.

CELLS of eukaryotes contain distinct subcellular compartments (organelles), delimited by characteristic membrane systems, which perform specialized functions within the cell. In photosynthetic leaf cells of higher plants, the most conspicuous organelles are the chloroplasts, occurring in a semi-autonomous fashion within the cell and containing their own genetic system and protein synthesis machinery, but relying on a close cooperation with the nucleocytoplasmic system for their development and biosynthetic activities¹. Most chloroplast proteins are encoded in the nuclear DNA and are the products of protein synthesis on cytoplasmic ribosomes, many as soluble precursors²⁻⁹ of higher relative molecular mass (M_r) which are then translocated through one or both of the plastid envelope mem-

branes, processed and assembled into their final organellar compartment or holoenzyme complex. *In vitro* reconstitution experiments using isolated chloroplasts have demonstrated that the uptake and processing of >100 nuclear-encoded, cytoplasmically synthesized precursors by chloroplasts occurs by an energy-dependent¹⁰, post-translational mechanism^{6,10-17}.

The most extensively characterized of these nuclear-encoded chloroplast proteins is the small subunit (SS) of ribulose 1,5-bisphosphate carboxylase. This polypeptide is synthesized on free cytoplasmic ribosomes as a precursor of M_r 20,000 with an amino-terminal extension or transit peptide of M_r ~5,000-6,000 (refs 6-7, 9). During or immediately after import of the precursor into the chloroplast, the transit peptide is proteolytically

Fig. 1 a, Construction of plasmids containing chimaeric genes encoding the TP-NPT-II fusion protein. A 1-kb *EcoRI/SphI* restriction fragment from pSR6, a pBR327 derivative containing the pea *ss3.6* gene³⁵, was purified from a 1% agarose gel. This fragment, which contains the promoter region, nucleotide sequences encoding the transit peptide and first Met codon of the mature SS polypeptide, was ligated into *EcoRI/BamHI*-cut pKm109/9 to replace the small *EcoRI/BamHI* fragment before the NPT-II-coding region. The plasmid pKm109/9 is a pBR322 derivative containing the *npt-II* gene of Tn5 lacking the 5'-non-translated region and the first Met codon⁴⁵. To fuse the 3'-overhanging end of the *SphI* restriction site with the 5'-overhanging end of the *BamHI* restriction site, a single-stranded oligonucleotide 5' GATCCATG 3', complementary to both protruding ends, was synthesized and added to the ligation mix⁵². After fusion, the *SphI* site is abolished but the *BamHI* site remains. The resulting plasmid, pGSSTneo1, was restricted with *SmaI* and a 700-base pair (bp) *PvuII* fragment, containing the transcription termination and polyadenylation signal from the octopine synthase gene (*ocs*)⁵³, was ligated into the site to ensure correct 3' transcription termination and processing. The intermediate pGSSTneo2 plasmid was then used in two different cloning steps. A, A 1,400-bp *BamHI* fragment from pUC4K (ref. 54) encoding the kanamycin resistance gene from Tn903 was isolated and cloned into the unique *BglII* restriction site of pGSSTneo2, yielding the plasmid pGSSTneo3. Kanamycin resistance is used as a marker to select for the co-integration of pGSSTneo3 with the Ti plasmid in *Agrobacterium*. B, A 200-bp *EcoRI/HindIII* fragment from pKm109/3 (ref. 45) containing the *lacUV5* promoter region, was exchanged for the small *EcoRI/HindIII* fragment of pGSSTneo2 to allow for TP-NPT-II fusion protein expression in *E. coli*. The resulting plasmid is pGLTneo1. Abbreviations: Ap^R, ampicillin resistance; Km^R, kanamycin resistance. Symbols: —, pBR322 sequence; neo, coding region for NPT-II; →, *lacUV5* promoter region; Δ, 3' end. Representation of the gene for the SS ribulose 1,5-bisphosphate carboxylase: ==, promoter and 5'-non-translated region; ///, sequence encoding the transit peptide; ■, exon; □, intron. **b**, Partial amino acid sequence comparison of the TP-NPT-II fusion protein and the precursor to the ribulose 1,5-bisphosphate carboxylase SS polypeptide. Partial amino acid sequences for the precursor to the SS polypeptide of ribulose 1,5-bisphosphate carboxylase encoded by the pea *ss3.6* gene³⁵ (upper line) and the TP-NPT-II fusion protein (lower line) are presented. The area near the processing site of the SS precursor and the fusion point for the TP-NPT-II fusion protein are shown. The arrow indicates the proteolytic processing site defined for the SS precursor. The amino acid residues derived from the original NPT-II protein are underlined and residues are numbered above the sequences with the first Met residue of the mature SS protein as amino acid number 1.



b

-57

Met...Ser Asn Gly Gly Arg Val Lys Cys Met Gln Val Trp Pro Pro Ile Gly Lys Lys ...

Met...Ser Asn Gly Gly Arg Val Lys Cys Met Asp Pro Ala Asn Leu Ala Trp Ile Glu ...

-1 ↓ +1

removed in two steps by a soluble protease¹⁸, yielding the mature SS polypeptide of M_r 15,000, which is then assembled with endogenous large subunit into the functional ribulose 1,5-bisphosphate carboxylase holoenzyme^{12,13}.

The transit peptides of post-translationally transported chloroplast proteins are characterized by a high proportion of basic amino acids; this is thought to be important in the interaction of the transit peptide with the chloroplast envelope¹⁹. Comparison of the transit peptide of SS precursors from various plant species show a high level of variation in amino acid sequences but a relatively strong conservation in the position of prolines and charged amino acid residues²⁰⁻²². This conservation may be of functional significance as the SS precursors from one plant species can be imported and processed correctly by the chloroplasts of another *in vitro*^{12,22} and *in vivo*²³.

The molecular basis of the post-translational translocation of polypeptides into chloroplasts and the signals involved are not

yet fully understood. In particular, the relative contributions of the transit peptide and the mature protein to the uptake and processing mechanism remain unknown. The transit peptide is presumably required for the translocation of the mature protein, consistent with the observation that the mature SS protein is not translocated into chloroplasts²⁴. We seek to find whether the transit peptide alone is sufficient to mediate the translocation of a foreign polypeptide into chloroplasts.

The genetic approach for the production of fusion proteins has successfully elucidated the mechanisms of protein transport and secretion in both prokaryotic and eukaryotic systems²⁵⁻³³. Chimaeric genes encoding fusion proteins have clarified the mechanism of protein import into yeast mitochondria³⁴. To examine the role of the transit peptide in the post-translational transport mechanism, we constructed a chimaeric gene which encodes a fusion protein consisting of the transit peptide of the precursor to the SS of ribulose 1,5-bisphosphate carboxylase

from pea³⁵ linked to the amino terminus of bacterial neomycin phosphotransferase II (NPT-II). This chimaeric gene was placed under the control of the pea small subunit (*ss3.6*) gene promoter^{35,36}, introduced into tobacco cells by *Agrobacterium*-mediated cell transformation^{23,36-44} and the fate of the fusion protein synthesized in these transformed cells studied. We also expressed the chimaeric gene in *Escherichia coli* under the control of the *lacUV5* promoter⁴⁵ and examined the fate of the fusion protein in *in vitro* reconstitution experiments using isolated intact chloroplasts.

Chimaeric gene construction

To elucidate the function of the transit peptide, we fused it with NPT-II, thus converting the bacterial enzyme into a novel 'precursor' polypeptide which can be tested for its ability to be post-translationally imported and processed by chloroplasts. The NPT-II protein was chosen because an enzymatic assay for *in situ* detection of NPT-II or NPT-II fusion proteins in non-denaturing polyacrylamide gels has been described⁴⁶ and is a useful method for distinguishing processed from unprocessed forms of the fusion protein.

Two plasmids have been constructed which contain chimaeric genes encoding the fusion protein TP-NPT-II (Fig. 1a). In the first plasmid, pGSSTneo3, the coding sequence for TP-NPT-II is under the control of the pea *ss3.6* promoter which directs expression of chimaeric genes in plant cells^{36,45}. This construction has been used to study the fate of the fusion protein *in vivo* in transformed tobacco cells. Another plasmid, pGLTneo1, was constructed to direct the synthesis of TP-NPT-II in *E. coli* under the control of the *lacUV5* promoter⁴⁵, to obtain sufficient quantities of the fusion protein for use in *in vitro* reconstitution experiments with isolated chloroplasts. The fusion protein encoded in both plasmids consists of the 57-amino acid transit peptide and the first methionine of the mature SS polypeptide encoded by the pea *ss3.6* gene³⁵, a 7-amino acid linker fragment, and the NPT-II lacking the first methionine⁴⁵ (263 amino acids). The amino acid sequences in the authentic SS precursor encoded by *ss3.6* and the fusion protein are compared in Fig. 1b; the Cys-Met cleavage site of the precursor to the SS is left intact in the TP-NPT-II fusion protein.

tp-npt-II gene transfer to plant cells

The *tp-npt-II* gene of pGSSTneo3 was introduced into the genome of plant cells by means of the vector pGV3851, a derivative of the *Agrobacterium* tumour-inducing (Ti) plasmid pTiC58 (ref. 47). The plasmid pGV3851 contains a deletion which removes several of the transfer (T)-DNA-encoded transcripts, including those involved in auxin production, but retains the gene involved in cytokinin synthesis. As a result, *Agrobacterium* harbouring pGV3851 induces shoot-forming tumours. The deleted portion of the T-DNA has been replaced by pBR322, which provides the homology for insertional recombination⁴⁸ of the pBR322 derivative pGSSTneo3.

The T-DNA of several kanamycin-resistant *Agrobacterium* exconjugants was examined by Southern hybridization analysis⁴⁹ to confirm that proper co-integration between pGSSTneo3 and the T-DNA of pGV3851 had occurred. The result obtained for one of these pGV3851::pGSSTneo3 exconjugants is shown in Fig. 2.

Stems of sterile tobacco seedlings were inoculated with this strain below the first internode after wounding with a needle. After 2-3 weeks, green shoot-forming tumours appeared. Axenic tissue was obtained by growing the transformed tissue *in vitro* on Murashige and Skoog medium⁵⁰ containing 500 µg ml⁻¹ of cefotaximum, an antibiotic to which ampicillin-resistant *agrobacteria* are sensitive. For propagation of the tissue, the sucrose concentration of the medium was reduced from 3 to 1%, to improve greening. The green tissues were able to grow on medium containing 200 µg ml⁻¹ kanamycin, showing that the *tp-npt-II* gene was present and functionally expressed. The

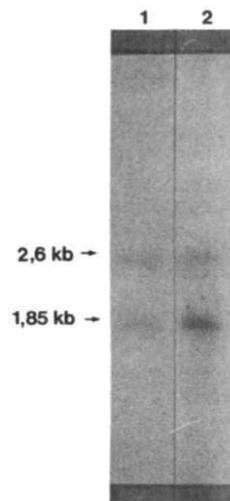


Fig. 2 Southern hybridization analysis of *Agrobacterium* and plant DNA. The autoradiogram confirms the presence and structure of the *tp-npt-II* chimaeric gene in co-integrate pGV3851::pGSSTneo3 DNA and in genomic DNA from transformed tobacco cells. Lane 1, total *Agrobacterium* DNA from pGV3851::pGSSTneo3 used in a reconstruction experiment to obtain a signal representing the presence of one copy per tobacco genome; lane 2, plant genomic DNA from tobacco callus transformed with pGV3851::pGSSTneo3. In both lanes two fragments hybridize with the specific probe: one fragment of 2.6 kb representing the *EcoRI/BamHI* fragment of pGSSTneo3 containing the *Km^R* gene of Tn903 and the SS promoter and transit peptide region; a second fragment of 1.85 kb representing the *BamHI/SalI* fragment of pGSSTneo3 containing the coding region of NPT-II and the OCS 3' end. Total *Agrobacterium* DNA⁵⁵ and plant genomic DNA from transformed callus tissue⁵⁶ were prepared and restricted with *EcoRI*, *BamHI* and *SalI*. Digest products were fractionated on a 1% agarose gel, transferred to nitrocellulose paper and hybridized with a ³²P-labelled probe specific for the promoter and coding region of the TP-NPT-II fusion protein (the probe was the smaller *EcoRI/SalI* fragment of pGSSTneo1; see Fig. 1a).

presence of the *tp-npt-II* gene was confirmed by Southern hybridization analysis⁴⁹ of genomic DNA from the transformed callus tissue (Fig. 2).

A parallel series of co-integration and transformation experiments (data not shown) provided tobacco tumours containing a second chimaeric gene, *nos-npt-II* (ref. 42) coding for the unaltered NPT-II protein under control of the promoter from the nopaline synthase gene^{37,44}, enabling us to study the fate of NPT-II itself in transformed cells.

Fate of the *tp-npt-II* gene product

The presence of NPT-II or active NPT-II fusion proteins in a given extract can be determined using an *in situ* enzymatic assay for phosphotransferase activity after gel electrophoresis. At the position where proteins with NPT-II activity migrate in a non-denaturing polyacrylamide gel, radiolabelled kanamycin phosphate is formed and detected by autoradiography (see Fig. 3 legend). The enzymatic assay on extracts of plant tissue not containing the NPT-II coding sequence in its genome reveals two bands of plant phosphotransferase or kinase activity (Fig. 3, lane 3, P.K.). These bands do not represent NPT-II activity as they can also be observed when no kanamycin is included in the enzymatic reaction (data not shown). The faster-migrating band is also found in chloroplast preparations from the same tissue (Fig. 3, lane 4). When a bacterial extract containing the TP-NPT-II fusion protein encoded in pGLTneo1 is mixed with plant extract, a major new band of NPT activity appears, migrating more slowly than NPT-II encoded by Tn5 and probably corresponding to the *bona fide* TP-NPT-II (Fig. 3, lanes 1, 2).

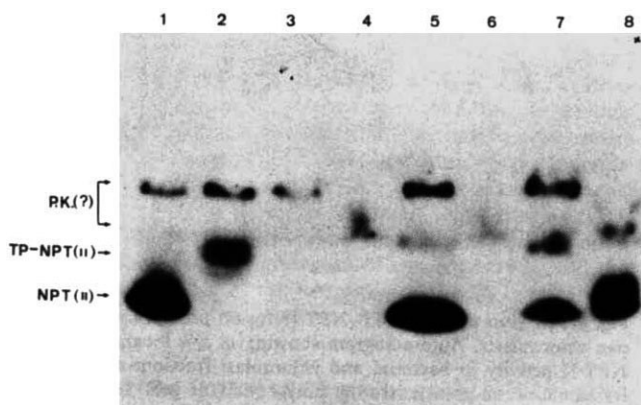


Fig. 3 Localization of NPT-II activity in chloroplasts of tobacco callus tissue. The autoradiogram shows the presence and mobility of NPT-II activity in bacterial extracts and cellular fractions following *in situ* localization on a 10% non-denaturing polyacrylamide gel⁴⁶. Lane 1, *E. coli* extracts containing NPT-II mixed with crude extract of green pGV3851-transformed tobacco tissue; lane 2, *E. coli* extract containing TP-NPT-II, mixed with crude extract of green pGV3851-transformed tobacco tissue; lane 3, crude extract from green pGV3851-transformed tobacco tissue; lane 4, intact chloroplasts from green pGV3851-transformed tobacco tissue; lane 5, crude extract from green pGV3851 :: pLGV23neo-transformed tobacco tissue; lane 6, intact chloroplasts from green pGV3851 :: pLGV23neo-transformed tobacco tissue; lane 7, crude extract from green pGV3851 :: pGSSTneo3-transformed tobacco tissue; lane 8, intact chloroplasts from green pGV3851 :: pGSSTneo3-transformed tobacco tissue. P.K.(?), non-specific band present in untransformed plant tissue, probably caused by the activity of plant kinase.

Methods. Green callus (3g) was homogenized by a few short bursts at low speed in a Waring Blendor in GR buffer (0.33 M sorbitol, 50 mM HEPES-KOH (pH 7.5), 1 mM MgCl₂, 1 mM MnCl₂, 1 mM Na₂-EDTA, 2 mM Na₂-EGTA, 1 mg ml⁻¹ isoascorbate, 0.5 mg ml⁻¹ bovine serum albumin). The homogenate was filtered through two layers of Miracloth and the filtrate centrifuged from 0–4,340g and braked in the shortest possible time. The crude chloroplast pellet was resuspended in a few millilitres of GR buffer. Intact chloroplasts were prepared from crude chloroplast pellets by sedimentation in Percoll density gradients⁵¹. Gradient-purified intact chloroplasts were washed with GR and lysed in 25 mM Tris-HCl (pH 7.5) containing 0.5% β -mercaptoethanol. Crude callus extracts were prepared by homogenizing 70 mg tissue in 70 μ l extraction buffer (1% β -mercaptoethanol; 50 mM Tris, pH 6.8, 0.13 mg ml⁻¹ leupeptin) and clearing the homogenate (2 min at 18,800g). Crude extracts of *E. coli* were prepared by sonication in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 25 mM NH₄Cl and 10 mM dithiothreitol (DTT)⁵⁷, followed by centrifugation to remove cellular debris. The assay for NPT-II activity is a modification of the *in situ* detection method⁴⁶. Samples diluted with a 10 $\times$ loading buffer (50% glycerol, 0.5% SDS, 10% β -mercaptoethanol, 0.005% bromophenol blue) were separated on a 10% (w/v) non-denaturing polyacrylamide gel. After electrophoresis, the gel was washed twice for 10 min with distilled water and equilibrated for 30 min in 2 $\times$ reaction buffer (100 mM Tris, pH 7.5, 50 mM MgCl₂, 400 mM NH₄Cl, 1 mM DTT). The gel was then transferred to a glass plate and overlaid with a 1% agarose gel containing 30 μ g ml⁻¹ kanamycin sulphate and 200 μ Ci [γ -³²P]ATP in 1 $\times$ reaction buffer. After 30 min at room temperature, the gel sandwich was covered with Whatman P81 phosphocellulose paper, two sheets of Whatman 3MM paper and a stack of blotting paper pressed by weight (1 kg) to allow binding of the phosphorylated kanamycin to the P81 paper in a Southern-type transfer. After 3 h the P81 paper was washed for 5 min with 500 ml hot water (80°C) and for 1 h several times with a 50 mM sodium phosphate buffer (pH 7.0). The P81 paper was dried and autoradiographed overnight using an intensifying screen to visualize the radiolabelled kanamycin formed at the position where the proteins with NPT-II activity migrate in the polyacrylamide gel.

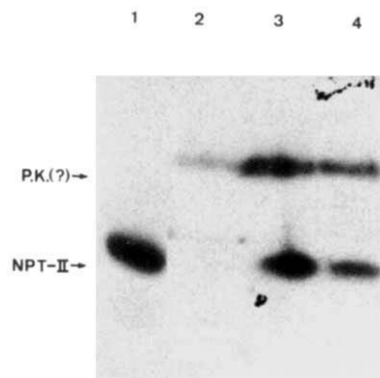


Fig. 4 Protection of the NPT-II activity present within chloroplasts of pGV3851 :: pGSSTneo3-transformed tobacco cells to protease treatment. Intact chloroplasts isolated from pGV3851 :: pGSSTneo3-transformed tobacco callus tissue were subjected to limited proteolytic digestion. The protease insensitivity of NPT-II activity associated with these chloroplasts was assayed. Lane 1, *E. coli* extract containing NPT-II; lane 2, intact chloroplasts isolated from green pGV3851 :: pGSSTneo3-transformed tobacco tissue and lysed before protease treatment; lane 3, non-protease-treated intact chloroplasts isolated from green pGV3851 :: pGSSTneo3-transformed tobacco tissue; lane 4, protease-treated intact chloroplasts isolated from green pGV3851 :: pGSSTneo3-transformed tobacco tissue; P.K.(?), see Fig. 3. Intact chloroplasts were prepared from greened tobacco callus tissue as described in the legend to Fig. 3. Protease treatment of isolated chloroplasts was carried out as described previously⁵¹.

The change in mobility results from a change in M_r and charge on addition of the transit peptide. Minor bands with higher mobility probably correspond either to degradation products of the fusion polypeptide or to smaller polypeptides translated from internal ATGs of the TP-NPT-II coding sequence.

Crude extracts from transformed tissue containing a *nos-npt-II* chimaeric gene have NPT-II activity. No detectable NPT-II activity is associated with intact chloroplasts isolated from the same tissue, however, suggesting that the product of this chimaeric gene lacks the information necessary to mediate its translocation into chloroplasts (Fig. 3, lanes 5, 6).

Crude extracts from tissue harbouring the *tp-npt-II* chimaeric gene also contain NPT-II activity (Fig. 3, lane 7), most of which is associated with chloroplasts (Fig. 3, lane 8). Moreover, the one neomycin-phosphorylating protein observed in both crude extracts and isolated chloroplasts migrates with the same mobility as the authentic NPT-II protein of Tn5, differing from the *E. coli* NPT-II fusion protein encoded by the *tp-npt-II* chimaeric gene (see also Fig. 4). Even after longer exposure of the autoradiogram, the NPT-II fusion protein was not found in preparations of plant tissue containing the *tp-npt-II* gene. These observations show that the NPT-II activity is concentrated in the chloroplast fraction and that the TP-NPT-II fusion protein is cleaved efficiently close to the fusion site, removing the transit peptide.

As the NPT-II protein is not normally imported into the chloroplasts, we sought to determine whether the NPT-II activity associated with the isolated chloroplast fraction is anchored only at the outside of the chloroplast, or whether it is really inside this organelle. Therefore, aliquots of chloroplasts isolated from pGV3851 :: pGSSTneo3-transformed tissue were subject to protease treatment⁵¹. Equal amounts of chloroplasts from protease-treated and non-treated preparations were fractionated and stromal fractions assayed for NPT-II activity. Most of the activity in non-treated chloroplasts remains in protease-treated chloroplasts until they are broken (Fig. 4). The slight decrease in activity observed probably results from plastid lysis rather than the lack of sequestering of the processed fusion protein.

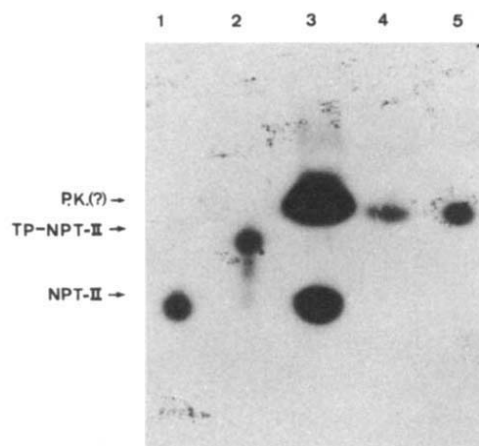


Fig. 5 Localization of NPT-II activity in the stromal fraction of chloroplasts isolated from pGV3851::pGSSTneo3-transformed tobacco tissue. Intact chloroplasts were isolated from pGV3851::pGSSTneo3-transformed callus tissue and fractionated into stromal and membrane fractions. The NPT-II activity associated with each of these fractions was assayed. Lane 1, *E. coli* extract containing NPT-II; lane 2, *E. coli* extract containing TP-NPT-II; lane 3, stromal fraction of chloroplasts isolated from green pGV3851::pGSSTneo3-transformed tobacco tissue; lane 4, membrane fraction of chloroplasts in lane 3; lane 5, wash of the lane 4 membrane fraction. P.K.(?), see Fig. 3. Intact chloroplasts were isolated from greened tobacco tissue as described in Fig. 3 legend. Chloroplasts washed twice with sorbitol-HEPES buffer and recovered by centrifugation were fractionated into stroma and membrane portions by resuspending plastids in 25 mM Tris-HCl (pH 7.5) containing 0.5% β -mercaptoethanol followed by centrifugation at 18,800g. Membrane fractions were washed twice and pelleted to remove residual stromal contamination. Wash fractions were routinely tested for residual NPT-II activity.

Finally, as the mature SS polypeptide is part of a soluble protein present in the stroma, we sought to determine whether the NPT-II activity associated with the isolated chloroplast fraction is sequestered also in the same suborganellar compartment. Therefore, chloroplasts from pGV3851::pGSSTneo3-transformed tissue were lysed by resuspension in a hypo-osmotic buffer and fractionated into stromal and membrane fractions which were assayed for NPT-II activity. All of the enzyme activity is located in the stromal rather than the membrane fraction of the chloroplasts (Fig. 5).

These results demonstrate clearly that the TP-NPT-II fusion protein is targeted to the chloroplast, translocated into the stroma and processed in a fashion similar to that of the SS polypeptide.

In vitro uptake and processing

As an alternative approach to studying the function of the transit peptide, we carried out a series of *in vitro* reconstitution experiments with isolated intact chloroplasts. This approach has been useful previously in the analysis of chloroplast translocation processes^{6,10-17}. Here we have adapted the method for *E. coli* fusion proteins.

Extracts containing the TP-NPT-II fusion protein were prepared from *E. coli* harbouring pGLTneo1 and aliquots incubated with chloroplasts isolated from pea leaves⁵¹. The chloroplasts were re-isolated from the incubation mix and washed until no TP-NPT-II activity was detectable in the supernatant. We used this preparation to determine whether NPT-II activity was associated with the stroma or membrane fraction. Before incubation with *E. coli* extracts containing TP-NPT-II, the stroma of chloroplasts from pea does not contain any phosphotransferase or kinase activity co-migrating with either the TP-NPT-II fusion polypeptide or authentic NPT-II (Fig. 6, lane 3). However, as

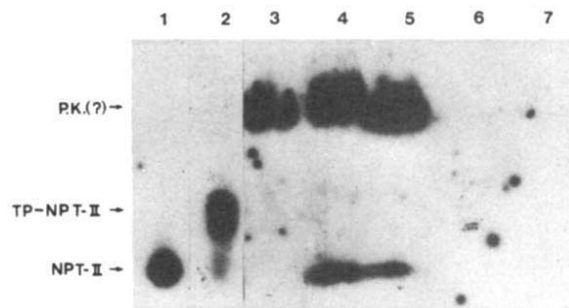


Fig. 6 *In vitro* uptake of TP-NPT-II fusion protein by isolated pea chloroplasts. Autoradiogram showing *in situ* localization of NPT-II activity in bacterial and chloroplast fractions following fractionation on non-denaturing polyacrylamide gels. Lane 1, *E. coli* harbouring pBR322::Tn5 (NPT-II); lane 2, *E. coli* harbouring pGLTneo1 (TP-NPT-II); lane 3, stromal fraction of pea chloroplasts before incubation with bacterial extracts; lane 4, stromal fraction of pea chloroplasts incubated with bacterial extracts containing the TP-NPT-II fusion protein; lane 5, stromal fraction of protease-treated pea chloroplasts (same amount as lane 4) incubated with bacterial extracts containing the TP-NPT-II fusion protein; lane 6, washed membrane fraction of the same chloroplasts as lane 5; lane 7, washed membrane fraction of the same chloroplasts as lane 4.

Methods. Intact chloroplasts were isolated from pea (*Pisum sativum*) leaves by sedimentation through Percoll density gradients⁵¹, washed, resuspended in sorbitol-HEPES buffer (50 mM HEPES-KOH, pH 7.5; 0.33 M sorbitol) and stored at 0°C. *In vitro* uptake into isolated chloroplasts was essentially as described⁵¹ except the incubation mix was modified for use with bacterial extracts. Uptake reactions (300 μ l final volume) contained intact chloroplasts (equivalent to 200–300 μ g chlorophyll) and 50 μ l bacterial extract (see Fig. 3 legend) in buffer containing 0.33 M sorbitol, 50 mM HEPES-KOH (pH 7.5), 1 mM MgCl₂, 1 mM Na₂-EDTA. Following incubation at 20–22°C in the light with gentle shaking for 1 h, chloroplasts were diluted with sorbitol-HEPES buffer and recovered by centrifugation at 4,340g. Chloroplasts washed twice with sorbitol-HEPES buffer and recovered by centrifugation were either fractionated immediately (see Fig. 4 legend) or subject to protease treatment as described previously⁵¹. Aliquots of samples were either assayed immediately for NPT-II, or stored at –80°C and assayed later.

observed earlier in tobacco, our assay conditions reveal a more slowly migrating endogenous kinase activity. After incubating the chloroplasts with bacterial extracts containing TP-NPT-II, the stromal fraction from the isolated organelles contains a considerable level of NPT-II activity whereas the membrane fraction does not (Fig. 6, lanes 4, 6). This NPT-II activity migrates like the original bacterial enzyme, indicating processing. To confirm that the activity is indeed associated with the stroma and not bound only to the external membrane of the chloroplast and liberated during the fractionation procedure, we examined the susceptibility to added protease. Most of the stromal NPT-II activity was protected against protease digestion, as the amount of activity recovered is similar to that found in non-treated chloroplasts (Fig. 6, lanes 4, 5). The membrane fraction of protease-treated chloroplasts was completely free of activity (Fig. 6, lane 7). *In vitro* uptake of the TP-NPT-II fusion protein is similar when intact chloroplasts from young expanding tobacco leaves are used (data not shown).

Our results demonstrate that the transit peptide of the precursor to the SS of ribulose 1,5-bisphosphate carboxylase can mediate uptake and processing of polypeptides other than the mature SS by chloroplasts in *in vitro* assay conditions. That uptake of the NPT-II protein by chloroplasts *in vitro* does not occur in the absence of the transit peptide (data not shown) is consistent with our *in vivo* observation that chloroplasts prepared from callus tissue transformed with *nos-npt-II* do not contain NPT-II activity.

Unlike previous *in vitro* uptake studies^{6,10-17} using wheat germ extracts for the synthesis of precursor polypeptides, we have used an *E. coli* expression system for the preparation of our fusion protein. As translocation of the fusion protein isolated from bacteria proceeds in the *in vitro* uptake system, we suggest that additional cytoplasmic factors are not required in the translocation mechanism. We cannot eliminate the possibility that such factors might be tightly bound to our chloroplast preparation or that equivalent factors may be provided by the *E. coli* extracts.

Discussion

We have examined the function of the transit peptide of the SS of ribulose 1,5-bisphosphate carboxylase by fusing it to the NPT-II protein and determining the fate of the fusion protein both *in vivo* in transformed tobacco cells and *in vitro* after incubation with isolated intact chloroplasts.

The results presented here demonstrate clearly that the NPT-II component of the TP-NPT-II fusion protein is translocated across the chloroplast envelope and finally located in the stroma. The requirement of the transit peptide for this process is shown by the failure of chloroplasts to detect uptake of NPT-II when the transit peptide is not fused to NPT-II. There is a high level of conservation in the amino acid sequence of the transit peptide and the mature protein near the processing site in the SS precursor of several plant species which was thought to reflect a possible functional role of these residues in the translocation process²⁰⁻²². The TP-NPT-II fusion protein, however, has no similarity in amino acid sequence to the SS precursor immediately following the transit peptide, which suggests that all of the sequence information required for translocation is in the transit peptide.

In normal plant growth conditions, the SS precursor is taken up rapidly and processed by chloroplasts and a large free pool of unprocessed precursor is not observed^{1,11}. In tobacco cells transformed with pGV3851::pGSSTneo3, the NPT-II activity migrates in our gel system with a similar electrophoretic mobility to the original NPT-II, indicating that the transit peptide has

been removed from the TP-NPT-II fusion protein. This processing is carried out presumably by the same stromal protease¹⁸ which removes the transit peptide from the precursor to the SS polypeptide. It is probable that processing occurs at the same Cys-Met site (Fig. 1b) used in the SS precursor and, therefore, that the transit peptide not only mediates translocation, but also site-specific processing. Furthermore, as in pGV3851::pGSSTneo3-transformed tobacco cells all of the observed NPT-II activity corresponds to the processed form of TP-NPT-II, we conclude that both translocation and processing occur efficiently for this novel 'precursor'.

The results presented here extend our knowledge and open the way for introduction of foreign gene products to specific plant cell organelles. It will be possible to discover whether similar chimaeric gene constructions could direct the transfer of proteins normally encoded by the chloroplast back into this organelle. Model systems of chloroplast-encoded genes important in basic research and agricultural application are readily available. The similarity between the results from *in vivo* and *in vitro* studies suggests also that the production in *E. coli* of fusion proteins composed of segments of nuclear-encoded organelle polypeptides and an enzymatic reporter is a powerful technique for the rapid analysis of the signals and processes involved in protein import by isolated organelles.

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- Ellis, R. J. A. *Rev. Pl. Physiol.* **32**, 111-137 (1981).
- Gillham, N., Boynton, J. & Chua, N.-H. *Curr. Topics Bioengng* **8**, 211-260 (1978).
- Gray, J. C. & Kekwick, R. G. O. *Eur. J. Biochem.* **44**, 491-503 (1974).
- Cashmore, A. J. *J. Biol. Chem.* **251**, 2848-2853 (1976).
- Apel, K. & Kloppstech, K. *Eur. J. Biochem.* **85**, 581-588 (1978).
- Highfield, P. E. & Ellis, R. J. *Nature* **271**, 420-424 (1978).
- Dobberstein, B., Blobel, G. & Chua, N.-H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1082-1085 (1977).
- Schmidt, G. W., Bartlett, S. G., Grossman, A. R., Cashmore, A. R. & Chua, N.-H. *J. Cell Biol.* **91**, 468-478 (1981).
- Cashmore, A. R., Broadhurst, M. K. & Gray, R. E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 655-659 (1978).
- Grossman, A. R., Bartlett, S. G. & Chua, N.-H. *Nature* **285**, 625-628 (1980).
- Chua, N.-H. & Schmidt, G. W. *J. Cell Biol.* **81**, 461-483 (1979).
- Chua, N.-H. & Schmidt, G. W. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6110-6114 (1978).
- Ellis, R. J., Smith, S. M. & Barraclough, R. in *Genome Organization and Expression in Plants* (ed. Leaver, C. J.) 321-335 (Plenum, New York, 1980).
- Schmidt, G. W., Bartlett, S. G., Grossman, A. R., Cashmore, A. R. & Chua, N.-H. in *Genome Organization and Expression in Plants* (ed. Leaver, C. J.) 337-351 (Plenum, New York, 1980).
- Grossman, A. R., Bartlett, S. G., Schmidt, G. W., Mullet, J. E. & Chua, N.-H. *J. Biol. Chem.* **257**, 1558-1563 (1982).
- Grossman, A. R., Bartlett, S. G., Schmidt, G. W. & Chua, N.-H. *Ann. N.Y. Acad. Sci.* **343**, 266-274 (1980).
- Chua, N.-H. & Schmidt, G. W. in *Photosynthetic Carbon Assimilation* (eds Siegelman, H. W. & Hind, G.) 325-347 (Plenum, New York, 1978).
- Robinson, C. & Ellis, R. J. *Eur. J. Biochem.* **142**, 343-346 (1984).
- Schmidt, G. W. *et al. J. Cell Biol.* **83**, 615-622 (1979).
- Brogie, R., Coruzzi, G., Lamppa, G., Keith, B. & Chua, N.-H. *Bio/technology* **1**, 55-61 (1983).
- Timko, M. P. & Cashmore, A. R. in *Plant Molecular Biology* (ed. Goldberg, R. B.) 403-412 (Liss, New York, 1983).
- Coruzzi, G., Brogie, R., Lamppa, G. & Chua, N.-H. in *Structure and Function of Plant Genomes* (eds Ciferri, O. & Dure, L. III) 47-59 (Plenum, New York, 1984).
- Brogie, R. *et al. Science* **224**, 838-843 (1984).
- Mishkind, N. L., Wessler, S. S. & Schmidt, G. W. *J. Cell Biol.* (in the press).
- Silhavy, T. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 5411-5415 (1977).
- Wolfe, P. B. & Wickner, W. *Cell* **36**, 1067-1072 (1984).
- Gething, M.-J. & Sambrook, J. *Nature* **300**, 598-603 (1982).
- Sabatini, D. D., Kreibich, G., Morimoto, T. & Adesnik, M. *J. Cell Biol.* **92**, 1-22 (1982).
- Emr, S. D. & Silhavy, T. J. *Cell Biol.* **95**, 689-696 (1982).
- Benson, S. A. & Silhavy, T. *Cell* **32**, 1325-1335 (1983).
- Guarente, L. & Ptashne, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2460-2464 (1981).
- Hall, M. N., Hereford, L. & Herskowitz, I. *Cell* **36**, 1057-1065 (1984).
- Lignappa, V. R., Chadez, J., Yost, C. S. & Hedpeth, J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 456-460 (1984).
- Douglas, M. G., Geiler, B. L. & Emr, S. S. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3983-3987 (1984).
- Cashmore, A. R. in *Genetic Engineering of Plants—An Agricultural Perspective* (eds Kosuge, T., Meredith, C. P. & Hollaender, A.) 29-38 (Plenum, New York, 1983).
- Herrera-Estrella, L. *et al. Nature* **310**, 115-120 (1984).
- Herrera-Estrella, L., Depicker, A., Van Montagu, M. & Schell, J. *Nature* **303**, 209-213 (1983).
- Fraley, R. T. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 4803-4807 (1983).
- Horsch, R. B. *et al. Science* **223**, 496-498 (1984).
- Bevan, M. W., Flavell, R. B. & Chilton, M.-D. *Nature* **304**, 184-187 (1983).
- Murai, N. *et al. Science* **222**, 476-482 (1983).
- Herrera-Estrella, L. *et al. EMBO J.* **2**, 987-995 (1983).
- Caplan, A. *et al. Science* **222**, 815-821 (1983).
- De Block, M. *et al. EMBO J.* **3**, 1681-1689 (1984).
- Reiss, B. thesis, Univ. Heidelberg (1982).
- Reiss, B., Sprengel, R., Will, H. & Schaller, H. *Gene* **30**, 217-223 (1984).
- Zambryski, P., Herrera-Estrella, L., De Block, M., Van Montagu, M. & Schell, J. in *Genetic Engineering, Principles and Methods* Vol. 6 (eds Setlow, J. & Hollaender, A.) 253-278 (Plenum, New York, 1984).
- Van Haute, E. *et al. EMBO J.* **2**, 411-418 (1983).
- Southern, E. M. *J. molec. Biol.* **98**, 503-518 (1975).
- Murashige, T. & Skoog, F. *Physiologia Pl.* **15**, 473-497 (1962).
- Bartlett, S. G., Grossman, A. R. & Chua, N.-H. in *Methods in Chloroplast Molecular Biology* (ed. Edelman, M.) 1081-1091 (Elsevier, Amsterdam, 1982).
- Lathe, R., Balland, A., Kohli, V. & Lecocq, J.-P. *Gene* **20**, 187-195 (1982).
- De Greve, H. *et al. J. molec. appl. Genet.* **1**, 499-512 (1982).
- Vieira, J. & Messing, J. *Gene* **19**, 259-268 (1982).
- Dhaese, P. *et al. EMBO J.* **2**, 419-426 (1983).
- Dellaporta, S. L., Wood, J. & Hicks, J. B. *Pl. molec. Biol. Rep.* **1**, 19-21 (1983).

Location of functional regions of acetylcholine receptor α -subunit by site-directed mutagenesis

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The availability of cloned cDNAs encoding the four subunits of the Torpedo acetylcholine receptor, which can be expressed to make functional receptors in Xenopus oocytes, has made possible a detailed investigation of the functions of the different structural components of the receptor. The functional analysis of receptors with α -subunits altered at specific sites by site-directed mutagenesis of the cDNA has allowed the location of specific regions of the α -subunit molecule involved in acetylcholine binding and forming a transmembrane ionic channel.

THE nicotinic acetylcholine receptor (AChR) mediating signalling at the vertebrate neuromuscular junction is the best-characterized ligand-gated channel protein. The AChR from the electric organ of *Torpedo californica* consists of four kinds of subunits assembled in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ (refs 1–3). The primary structures of all these four subunits^{4–6} and the α -, β - and γ -subunits of the mammalian muscle AChR^{7–9} have been elucidated by cloning and sequencing cDNAs or genomic DNA encoding these polypeptides; cDNA sequences for the γ -subunit of the *T. californica* AChR¹⁰ and the α -subunit of the *T. marmorata* AChR^{11,12} have been reported also. The four subunits have marked sequence homology and are similar in hydrophilicity profile and predicted secondary structure, thus being oriented most probably in a pseudosymmetric fashion across the membrane. The carboxy-terminal half of each subunit molecule contains four strongly hydrophobic segments^{4–10,12} (M1–M4) consisting exclusively of uncharged amino acid residues. These segments probably traverse the membrane, forming α -helical structures, and may be involved in the ionic channel. Interestingly, the polar uncharged side chains in these segments are clustered largely on one side of an α -helix. On the basis of the primary structure data, it has been proposed subsequently that each subunit contains a fifth transmembrane segment, located between the hydrophobic segments M3 and M4. This segment is markedly amphipathic and has both charged and uncharged polar residues on one side of an α -helix and non-polar residues on the opposite side^{13,14}, thus presumably forming the channel lining. The region lying between segment M3 and the amphipathic segment has been assigned to the cytoplasmic side of the membrane, whereas the amino-terminal half of each subunit molecule, which contains one or more potential *N*-glycosylation sites^{4–12}, has been assigned to the extracellular side of the membrane. The amino-terminal half of the α -subunit molecule has four cysteine residues^{4,7,11,12} which may take part in the disulphide bridge in close proximity to the acetylcholine (ACh) binding site¹⁵.

We have shown recently that the cloned cDNAs encoding the four subunits of the *T. californica* AChR direct the synthesis of the functional receptor¹⁶. Studies on the expression of AChR mutants with the α -subunit altered by site-directed mutagenesis of the cDNA have now allowed the location of functional regions of the subunit molecule.

Expression system

Previously, we used a combined expression system consisting of COS monkey cells and *Xenopus* oocytes¹⁶. We report here the synthesis of AChR subunit-specific mRNAs *in vitro*, using the bacteriophage SP6 promoter^{17,18} linked with each of the subunit cDNAs including the poly(A) tail. Transcription of these

recombinant plasmids by SP6 polymerase generated pure preparations of the subunit-specific mRNAs in relatively large quantities (Fig. 1a). After capping, the α -, β -, γ - and δ -subunit-specific mRNAs were combined and injected into *Xenopus* oocytes. The four AChR subunit polypeptides synthesized in the oocytes were indistinguishable in electrophoretic mobility on an SDS-polyacrylamide gel from those synthesized in oocytes injected with *T. californica* electroplax poly(A)⁺ RNA (Fig. 1b). The amounts of the radiolabelled AChR subunit polypeptides, as well as the α -bungarotoxin binding activity in oocyte extracts and the ACh sensitivity of the oocytes, increased approximately in proportion to the concentration of the mRNAs injected. (The total mRNA concentration used here ranged from 4 to 250 ng μ l⁻¹; the average volume injected was ~20 nl per oocyte.)

The injected oocytes were examined for response to ACh applied ionophoretically by recording the intracellular potentials or the membrane currents under voltage clamp¹⁶. The atropine-resistant and (+)tubocurarine-sensitive nature of the observed ACh response conformed to that of the nicotinic AChR synthesized in oocytes injected with *Torpedo* electroplax poly(A)⁺ RNA¹⁹ or with the AChR subunit-specific mRNAs produced in COS cells¹⁶. In our present studies, the ACh sensitivity²⁰ occasionally exceeded 10,000 mV μ C⁻¹, which is two orders of magnitude higher than that obtained previously¹⁶. Unexpectedly, the membrane potential-dependence of the ACh response in most of the oocytes with high sensitivities (> 100 mV μ C⁻¹) differed from that expected for the nicotinic AChR. The ACh current had multiple components (Fig. 2a) and was reversed in polarity at membrane potentials (–30 to –15 mV) more negative than those (–5 to –1 mV) reported previously for the nicotinic AChR synthesized in oocytes^{16,19} (Fig. 2c). Furthermore, the reversal potential was apparently Cl⁻-dependent (shown by its alteration after intracellular Cl⁻ injection or decreased external Cl⁻ concentration). When external Ca²⁺ was removed or replaced by Mg²⁺ (5 mM), the multiplicity of the ACh current disappeared (Fig. 2b) and the reversal potential agreed with that of the nicotinic AChR (Fig. 2c). Therefore, we conclude that a Ca²⁺ influx associated with activation of the AChR²¹ may trigger a transient increase in the Cl⁻ conductance of the oocyte membrane^{22,23}, thereby initiating an additional Cl⁻ current mixed with the cationic ACh current. A similar Cl⁻ contribution to the ACh current was observed also in the oocytes injected with *T. californica* electroplax poly(A)⁺ RNA when their ACh sensitivity was higher than 100 mV μ C⁻¹. Because of uncertainty in the extent of the Cl⁻ contribution to the ACh response, the ACh sensitivity of oocytes with the wild-type AChR as well as with the AChR mutants was measured both in the presence and absence of Ca²⁺. The results obtained in the two conditions were consistent with our general con-

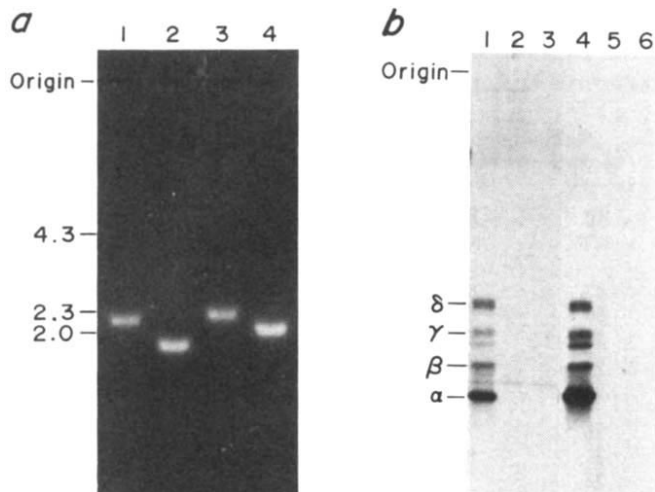


Fig. 1 Expression of the AChR subunit cDNAs by transcription *in vitro* (a) followed by translation in *Xenopus* oocytes (b). a, The AChR subunit-specific mRNAs synthesized *in vitro* were denatured with 1 M glyoxal and 50% dimethyl sulphoxide³¹ and samples (0.6 μ g each) of the α - (lane 1), β - (lane 2), γ - (lane 3) and δ -subunit-specific mRNA (lane 4) were electrophoresed on a 1.5% agarose gel in 10 mM sodium phosphate buffer, pH 7.0. RNA was visualized by staining with ethidium bromide. The size markers used were the *Hind*III cleavage products of phage λ DNA; sizes in kilobases (kb). The estimated sizes of the α -, β -, γ - and δ -subunit-specific mRNAs agree with the expected sizes (~ 2.1 , ~ 1.8 , ~ 2.2 and ~ 2.0 kb, respectively). b, *Xenopus laevis* oocytes were injected with the capped α -, β -, γ - and δ -subunit-specific mRNAs combined in a molar ratio of 2:1:1:1 (total mRNA concentration 100 ng μ l⁻¹) or with *T. californica* electroplax poly(A)⁺ RNA (1 μ g μ l⁻¹). After incubation of the oocytes for 2 days in the presence of L-³⁵S-methionine (1,370 Ci mmol⁻¹; final concentration, 0.8 mCi ml⁻¹), cell extracts were prepared from 38 oocytes (see ref. 16 for experimental details). The AChR was immunoprecipitated with rabbit antiserum to the *T. californica* AChR and protein A-Sepharose and was electrophoresed on an SDS/10% polyacrylamide gel³². The fluorogram³³ of the electrophoretic analysis is shown. Lane 1, immunoprecipitate derived from oocytes injected with *T. californica* electroplax poly(A)⁺ RNA (corresponding to 10 oocytes); lane 2, control for lane 1 (immunoprecipitate obtained in the presence of an excess of the purified³⁴ *T. californica* AChR); lane 3, another control for lane 1 (immunoprecipitate obtained with non-immunized rabbit serum); lane 4, immunoprecipitate derived from oocytes injected with the mRNAs synthesized *in vitro* (corresponding to 0.7 oocyte); lane 5, control for lane 4 (like lane 2); lane 6, another control for lane 4 (like lane 3). The positions to which the α -, β -, γ - and δ -subunits of the purified *T. californica* AChR migrated are indicated. The additional polypeptide found beneath the γ -subunit may have resulted from different glycosylation or proteolytic modification of the γ -subunit *in vivo* or *in vitro*^{1,16}.

Methods. The *Bgl*II site in the 5'-non-coding region of the AChR α -subunit cDNA in the plasmid pKCR α 1 (ref. 16) was deleted by cleavage with *Bgl*II followed by treatment with T4 DNA polymerase in the presence of the four deoxyribonucleoside triphosphates (dNTPs). The resulting plasmid pKCR α 3 and the plasmid pKCR γ 1 (ref. 16) were cleaved with *Hind*III and the fragments containing the AChR subunit cDNA inserted into the *Hind*III site of the plasmid pSP64 (ref. 18) in the same orientation as the SP6 promoter to yield the plasmids pSP α and pSP γ , respectively. The plasmids pKCR β 1 and pKCR δ 1 (ref. 16) were digested partially with *Eco*RI, treated by T4 DNA polymerase with the four dNTPs and cleaved completely with *Eco*RI. The resulting DNA fragments containing the AChR subunit cDNA were ligated with the 3.0-kb pair fragment of pSP64, prepared by successive treatments with *Hind*III, T4 DNA polymerase with the four dNTPs and *Eco*RI, to yield the plasmids pSP β and pSP δ , carrying the β - and δ -subunit cDNA, respectively, in the same orientation as the SP6 promoter. The AChR subunit-specific mRNAs were synthesized *in vitro*, using *Sma*I-cleaved pSP α , *Eco*RI-cleaved pSP β , *Pst*I-cleaved pSP γ and *Eco*RI-cleaved pSP δ as templates and were then capped *in vitro* with vaccinia virus mRNA guanylyl transferase; the procedures used were as described previously¹⁷.

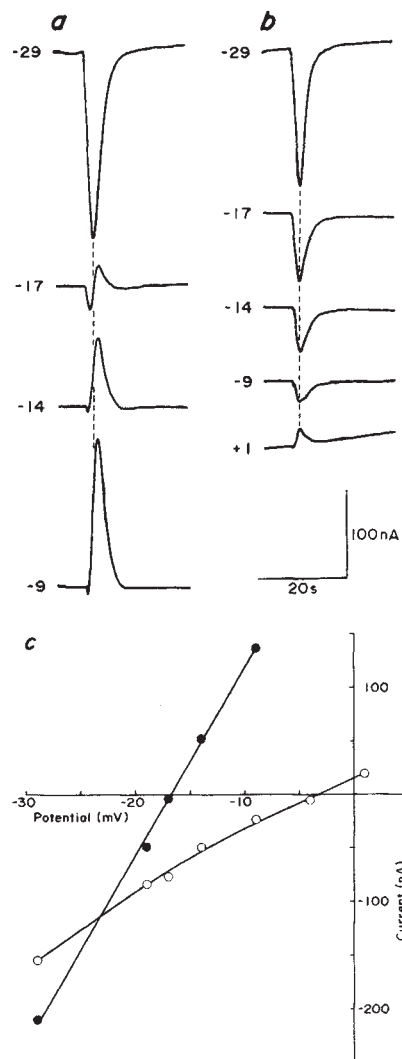


Fig. 2 Membrane potential-dependence of ACh responses recorded from a *Xenopus* oocyte injected with the four AChR subunit-specific mRNAs synthesized *in vitro*. The oocyte was incubated for 3 days in the conditions described previously¹⁶. Recordings were made at room temperature in Ringer's solution (a) and in nominally Ca²⁺-free Ringer's solution (b), both containing 1 μ M atropine. ACh was applied by constant ionophoretic current pulses (22 nA in intensity and 1 s in duration; backing current, 12 nA) through a pipette filled with 1 M ACh. Currents (downward deflection indicating inward current) were recorded under voltage clamp at different levels of the membrane potential (mV) as indicated on the left side of each record. c, Relations between current (inward current being shown as negative current) and membrane potential observed in Ringer's solution (●) and in the Ca²⁺-free solution (○). The currents plotted were measured at a fixed time as indicated by dashed lines in a and b.

clusions. The reversal potentials of all the responsive AChR mutants, examined in the absence of Ca²⁺, were in the normal range.

Deletion mapping

To locate the segments of the AChR α -subunit involved in the ionic channel, we introduced internal deletions to various loci of the cDNA corresponding to the carboxy-terminal half of the subunit molecule (Fig. 3). Each of the α -subunit-specific mRNAs synthesized with the cDNA templates with internal deletions, combined with the wild-type β -, γ - and δ -subunit-specific mRNAs, was injected into *Xenopus* oocytes. The oocytes were examined for the content of the AChR subunit polypeptides and for the functional properties of the AChR formed. The amounts of the AChR subunits were estimated by labelling the

Table 1 Functional properties of AChR mutants with altered α -subunit

α -Subunit	Residue change		^{125}I - α -Bungarotoxin binding			Response to ACh	
	From	To	Activity (%)	% Inhibition by carbamylcholine 0.5 mM	10 mM	No. of responsive oocytes/no. of oocytes tested	Mean ACh sensitivity (%)
Wild type			100	38	80	82/82	100
$\alpha\Delta 224$ -237	LFSFLTGLVF YLPT	PSS	0.5	NT	NT	0/15	ND
$\alpha\Delta 249$ -257	VLLSLTVFL	SS	17	47	87	0/15	ND
$\alpha\Delta 279$ -284	LFTMIF	PSS	8	50	80	0/15	ND
$\alpha\Delta 316$ -322	FIDTIPN	RAR	60	39	86	28/29	31
$\alpha\Delta 327$ -334	STMKRASK	RAR	80	44	82	14/14	70
$\alpha\Delta 334$ -344	KEKQENKIFA D	A	71	44	82	29/29	55
$\alpha\Delta 344$ -352	DDIDISDIS	ELA	101	36	79	14/14	122
$\alpha\Delta 353$ -357	GKQVT	R	58	37	82	29/29	142
$\alpha\Delta 355$ -389	QVTGEVIFQT PLIKNPDKVS AIEGVKYIAE HMKSD	PSS	45	38	77	14/14	3
$\alpha\Delta 363$ -367	QTPLI	PSS	83	39	77	14/14	101
$\alpha\Delta 366$ -371	LIKNDP	SS	81	34	81	29/29	31
$\alpha\Delta 366$ -389	LIKNDPKSA IEGVKYIAEH MKSD	SS	37	38	78	2/14	0.1
$\alpha\Delta 371$ -377	DVKSAIE	A	44	38	81	4/14	0.2
$\alpha\Delta 376$ -383	IEGVKYIA	EL	43	46	84	0/14	ND
$\alpha\Delta 376$ -389	IEGVKYIAEH MKSD	SS	36	37	78	0/14	ND
$\alpha\Delta 382$ -389	IAEHMKSD	TSS	29	40	81	0/14	ND
$\alpha\Delta 389$ -395	DEESSNA	EL	48	38	80	29/29	65
$\alpha\Delta 394$ -401	NAAEEWKY	TSS	40	54	89	0/42	ND
$\alpha\Delta 409$ -420	ILLCVFMLIC II	EL	23	38	77	0/29	ND
$\alpha\Delta 428$ -434	GRLIELS	R	80	39	83	27/27	15
$\alpha 128\text{S}$	C	S	0.08	NT	NT	0/14	ND
$\alpha 142\text{S}$	C	S	ND	NT	NT	0/14	ND
$\alpha 192\text{S}$	C	S	39	8	27	0/14	ND
$\alpha 193\text{S}$	C	S	28	13	53	0/14	ND
$\alpha 222\text{S}$	C	S	79	36	83	28/28	107
$\alpha 141\text{D}$	N	D	0.09	NT	NT	0/14	ND

Oocytes were injected with the α -subunit-specific mRNA having various mutations, combined with the wild-type β -, γ - and δ -subunit-specific mRNAs in a molar ratio of 2:1:1:1; the total mRNA concentration was 100 or 250 $\mu\text{g l}^{-1}$ for assay of ^{125}I - α -bungarotoxin binding and 100 ng l^{-1} for assay of ACh potentials. After incubation of the oocytes for 3 days in the conditions described previously¹⁶, the ^{125}I - α -bungarotoxin binding activity in cell extracts was assayed in the presence and absence of 0.5 mM and 10 mM carbamylcholine. The assay was carried out as described previously¹⁶, using 2 nM toxin and rabbit antiserum to the *T. californica* AChR; when 0.2 nM toxin was used for the assay, the extent of toxin binding by the AChR mutants (except for the mutants with $\alpha\Delta 224$ -237, $\alpha 128\text{S}$, $\alpha 142\text{S}$ or $\alpha 141\text{D}$) as well as by the wild-type AChR was 37-54% of the activity measured with 2 nM toxin, suggesting that the apparent binding affinity for ^{125}I - α -bungarotoxin was not affected substantially by the mutations. ACh potentials were recorded at a holding potential of ~ -60 mV in Ringer's solution in which CaCl_2 was replaced by 5 mM MgCl_2 ; see Fig. 2 legend for further details. ND, not detectable; NT, not tested. Amino acid residues are given in the one-letter code. The toxin binding activity of the wild-type AChR was 14-83 and 34-114 fmol per oocyte, respectively, when the mRNA concentration used for injection was 100 and 250 ng l^{-1} . Our detectable limit was ~ 0.01 fmol per oocyte. The mean ACh sensitivity of the wild-type AChR ranged from 150 to 1,100 $\text{mV } \mu\text{C}^{-1}$. Our detectable limit was ~ 0.2 $\text{mV } \mu\text{C}^{-1}$.

polypeptides with ^{35}S -methionine and isolating them by immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis. Densitometric scanning of the fluorograms (Fig. 4a-e) showed that for all AChR mutants with a deletion in the α -subunit, there were sufficient concentrations of the four subunit polypeptides (at least 10% of the wild type) to allow assay of the functional parameters for the AChR.

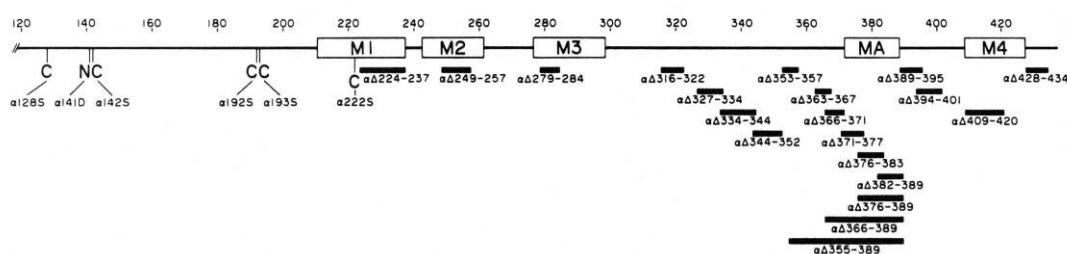
The effects of the various deletions in the AChR α -subunit on the ^{125}I - α -bungarotoxin binding activity and the membrane potential response to ACh are shown in Table 1. Inhibition of α -bungarotoxin binding by carbamylcholine was used as a measure of the binding affinity for the agonist. The toxin binding activities of most of the AChR mutants with a deletion in the α -subunit were comparable with the wild-type activity, whereas the AChR mutant with the α -subunit with a deletion in segment M1 ($\alpha\Delta 224$ -237) had markedly reduced activity. The relatively low toxin binding activities of some other AChR mutants (for example, $\alpha\Delta 279$ -284) can roughly be accounted for by the observed decreases in the amount of the α -subunit responsible

for α -bungarotoxin binding^{16,24,25}.

The toxin binding activity of the AChR mutant with $\alpha\Delta 224$ -237, however, was much lower than expected from the concentration of α -subunit present ($\sim 30\%$ of the wild type). (Although the amount of the δ -subunit in this mutant was $\sim 10\%$ of the wild type, this subunit is not essential to toxin binding¹⁶.) Because segment M1, some of which is deleted in the α -subunit mutant $\alpha\Delta 224$ -237, is adjacent to the extracellularly located amino-terminal half of the subunit molecule, this mutation may result in a conformational change in the region encompassing the toxin binding site. The apparent binding affinity for the agonist was not affected significantly in the AChR mutants with a deletion in the α -subunit, except for the mutant with $\alpha\Delta 394$ -401, which showed reproducibly an apparent K_D value two- to four-fold lower than that of the wild type (Fig. 5a); the mutant with $\alpha\Delta 224$ -237 exhibited too little toxin binding to allow measurement of the inhibition by carbamylcholine.

All the oocytes injected with the four wild-type subunit-specific mRNAs responded to ACh (mean sensitivities, 150-

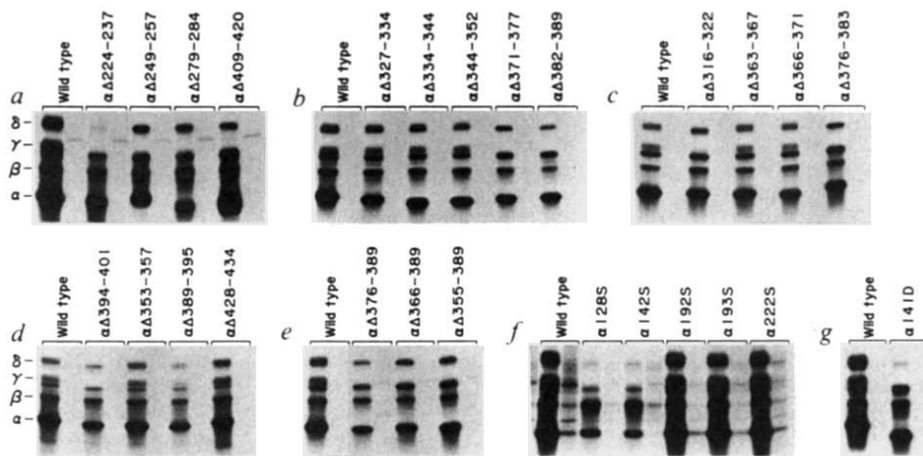
Fig. 3 Mutations introduced into the *T. californica* AChR α -subunit polypeptide. The putative transmembrane segments M1, M2, M3, MA and M4 are represented by open boxes and the amino acid numbers of the AChR α -subunit⁴ are given above the diagram. The positions of deletions are indicated by bars and those of the cysteine and asparagine residues subjected to point mutations by the one-letter code; the mutant numbers indicate the number(s) of the amino acid residue(s) deleted or altered.



of deletions are indicated by bars and those of the cysteine and asparagine residues subjected to point mutations by the one-letter code; the mutant numbers indicate the number(s) of the amino acid residue(s) deleted or altered.

Methods. The deletion mutants of the AChR α -subunit cDNA were constructed as follows: The plasmid pSP α was cleaved with either *Bgl*II or *Pst*I, treated with *Bal*31 exonuclease, ligated with the *Sac*I linker CGAGCTCG and cleaved with *Sac*I and *Hind*III. DNA fragments containing a 5' portion of the α -subunit cDNA were ligated with the 3.0-kb *Hind*III/*Sac*I fragment from pSP64 to yield a series of deletion mutants (pSP α ND). The 2.1-kb *Eco*RI fragment from pKCR α 1 was inserted into the *Eco*RI site of pSP64. pSP α , containing the α -subunit cDNA in the same orientation as the SP6 promoter, cleaved with *Bgl*II or *Pst*I, treated with *Bal*31 exonuclease, ligated with the *Sac*I linker and cleaved with *Sac*I and *Eco*RI. DNA fragments containing a 3' portion of the α -subunit cDNA were ligated with the 3.0-kb *Sac*I/*Eco*RI fragment from pSP64 to yield a series of deletion mutants (pSP α CD). Additional deletion mutants were constructed similarly by *Bal*31 exonuclease digestion of the α -subunit cDNA from the *Hae*III (1, 284), *Hha*I (809) and *Hpa*II (494) sites⁴. The end point of each deletion was determined by DNA sequencing³⁵ after subcloning the *Hind*III/*Sac*I fragment from pSP α ND and pSP α CD into the vector M13mp11 (ref. 36). The *Sac*I/*Eco*RI fragment containing a 5' portion of the α -subunit cDNA, derived from pSP α ND, was ligated with the *Sac*I/*Eco*RI fragment containing a 3' portion of the α -subunit cDNA, derived from pSP α CD, to produce a plasmid (pSP α Δ) carrying the α -subunit cDNA with an internal deletion. Some of the plasmids obtained were cleaved by *Sac*I, treated by T4 DNA polymerase with the four dNTPs and ligated to adjust the translational reading frame in the α -subunit cDNA. All the constructions were confirmed by sequencing³⁵ the region encompassing the deletions. The point mutations were introduced²⁹ into the AChR α -subunit cDNA as follows: the 1.8-kb *Hind*III fragment containing the α -subunit cDNA, derived from pKCR α 2 (ref. 16), was inserted into the *Hind*III site of the vector M13mp8 (ref. 36) in the same orientation as the lactose operon. The mutagenic oligodeoxynucleotides, synthesized by the triester method³⁷, were 5'-AA-TTTCATATAGCTTT-3' (α 128S), 5'-CATAGTGCTATTTGTT-3' (α 142S), 5'-AGGACAGCTGGTATAAT-3' (α 192S), 5'-GTCAG-GACTGCAGGTAT-3' (α 193S), 5'-AAGCAGACTAGGAATGA-3' (α 222S) and 5'-AGTGCAATCTTGTGAT-3' (α 141D). To ensure that no other mutation than that required occurred during mutagenesis, we sequenced³⁵ the entire region extending from the *Pvu*II (109) site to the *Bgl*II (942) site of the α -subunit cDNA inserted in the recombinant phage. The 0.8-kb *Pvu*II/*Bgl*II fragment from the replicative form DNA of the recombinant phage with a point mutation was ligated with the 4.3-kb *Pvu*II/*Bgl*II fragment from pSP α . For further experimental details, see ref. 38.

Fig. 4 Subunit polypeptides of AChR mutants synthesized in *Xenopus* oocytes. The derivatives of the plasmid pSP α with an internal deletion in the α -subunit cDNA and those having a point mutation were cleaved with *Eco*RI and *Sma*I, respectively, and were used as templates for transcription *in vitro* by SP6 polymerase to prepare the mRNAs coding for the α -subunit mutants. Oocytes were injected with the α -subunit-specific mRNA with various mutations, combined with the wild-type β -, γ - and δ -subunit-specific mRNAs in a molar ratio of 2:1:1:1 (total mRNA concentration, 100 ng μ l⁻¹). The subsequent procedures were essentially as described in Fig. 1 legend. *a-g*, Fluorograms of electrophoretic analysis of the indicated AChR mutants and the wild-type AChR; two lanes are presented for each sample, bracketed together, of which the left lane shows the immunoprecipitate obtained with rabbit antiserum to the *T. californica* AChR and the right lane a control immunoprecipitate obtained with non-immunized rabbit serum (*a-e* and *g*) or obtained with rabbit antiserum to the AChR in the presence of an excess of the purified *T. californica* AChR (*f*). *a*, *f* and *g* have been overexposed to show the faint bands. Note that some of the α -subunit mutants, for example α Δ249-257, had an electrophoretic mobility different from that expected from their molecular weights.



1,000 mV μ C⁻¹ in Ca²⁺-free medium, depending apparently on the conditions of the oocytes used). In contrast, the AChR mutants with the α -subunit deletion in segment M1 (α Δ224-237), M2 (α Δ249-257), M3 (α Δ279-284) or M4 (α Δ409-420) failed to respond to ACh. Some of the AChR mutants with the α -subunit deletion between M3 and M4 were hardly or not at all responsive to ACh (α Δ355-389, α Δ366-389, α Δ371-377, α Δ376-383, α Δ376-389, α Δ382-389 and α Δ394-401), whereas the others had an essentially normal response (α Δ316-322, α Δ327-334, α Δ334-344, α Δ344-352, α Δ353-357, α Δ363-367, α Δ366-371 and α Δ389-395). One AChR mutant with the α -subunit having a deletion on the carboxy-terminal side of M4 (α Δ428-434) was also responsive to ACh.

The short deletions between residues 316 and 371 of the α -subunit thus did not impair substantially the functional

properties of the AChR, nor did they affect appreciably the content of the AChR subunit polypeptides. Hence, the proposal that the segment of residues 334-354 is involved in the ionic channel²⁶ seems unlikely. The dispensable segment found corresponds to most of the putative cytoplasmic region. It remains possible that this segment is responsible for other features of the AChR, such as the clustering of receptor molecules or the phosphorylation of the receptor. In contrast, the short deletions in the four hydrophobic segments (M1-M4) of the α -subunit abolished the ACh response, but did not alter the apparent binding affinity for the agonist. (The effect of the deletion in segment M1 on the agonist binding could not be studied.) This suggests that these segments take part directly or indirectly in the formation of the ionic channel. The possibility that the deletion in M1 also affects the agonist binding cannot be

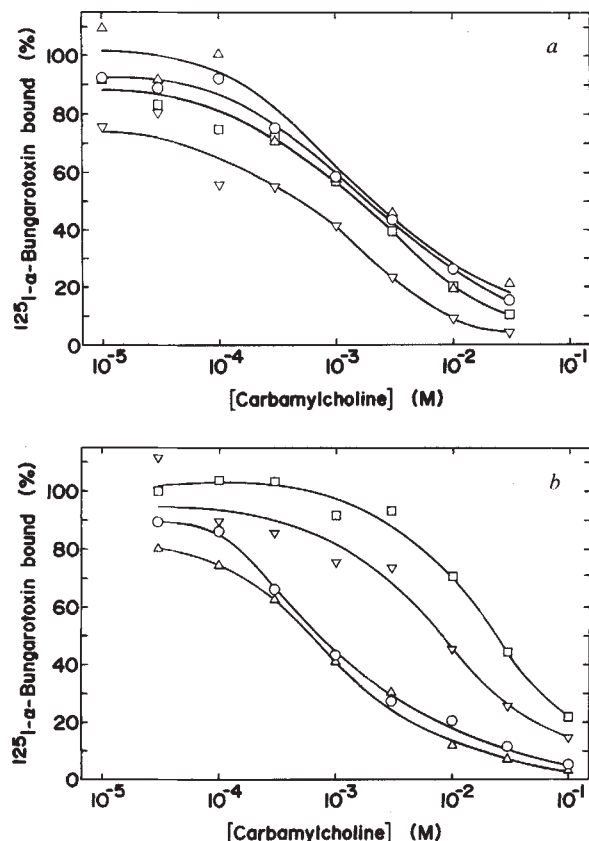


Fig. 5 Effect of carbamylcholine on ^{125}I - α -bungarotoxin binding by AChR mutants. *a*, AChR mutants with $\alpha\Delta 389-395$ (Δ), $\alpha\Delta 394-401$ (∇) or $\alpha\Delta 428-434$ ($\square$) and wild-type AChR ($\circ$). *b*, AChR mutants with $\alpha 192\text{S}$ ($\square$), $\alpha 193\text{S}$ (∇) or $\alpha 222\text{S}$ (Δ) and wild-type AChR ($\circ$). The apparent K_D values for ^{125}I - α -bungarotoxin¹⁶ of these mutants were essentially the same as that of the wild type ($\sim 0.2\text{ nM}$). Oocytes were injected with the α -subunit-specific mRNA with the indicated mutations, combined with the wild-type β -, γ - and δ -subunit-specific mRNAs, as described in Fig. 4 legend, and were incubated for 3 days as specified previously¹⁶. The effects of increasing concentrations of carbamylcholine on the ^{125}I - α -bungarotoxin binding activity in cell extracts were examined as described previously¹⁶ using 2 nM toxin. The toxin binding activities were not affected by the presence of 0.03 M and 0.1 M NaCl instead of carbamylcholine chloride.

excluded. The amounts of the AChR subunit polypeptides found for these mutants ($\geq 10\%$ of the wild type) should be sufficient for the receptor function because we found that similar or even smaller amounts of the wild-type polypeptides elicited a clear response to ACh.

The positions of the deletions in the α -subunit of the AChR mutants showing little or no ACh response ($\alpha\Delta 355-389$, $\alpha\Delta 366-389$, $\alpha\Delta 371-377$, $\alpha\Delta 376-383$, $\alpha\Delta 376-389$ and $\alpha\Delta 382-389$) and of the responsive mutants with $\alpha\Delta 366-371$ or $\alpha\Delta 389-395$ indicate that the segment of residues 372-388 (segment MA) is required for the receptor function. Remarkably, this segment coincides nearly with the amphipathic segment^{13,14}. Furthermore, all the AChR mutants with the α -subunit lacking part or all of segment MA had essentially normal apparent binding affinity for the agonist and sufficient contents of the subunit polypeptides. Thus, our results suggest that this presumably α -helical amphipathic segment spans the membrane and is involved in the ionic channel, probably constituting its inner wall. This supports the view that there are five transmembrane segments in each subunit^{13,14}. The finding that the AChR mutant with $\alpha\Delta 355-389$, in which the entire segment MA is deleted, has a slight but unambiguous response to ACh seems to contradict our conclusion, but in the α -subunit mutant $\alpha\Delta 355-389$ the segment of residues 340-354, which takes over the original position of segment MA, shows an amphipathic α -helical

character^{13,26} (to a lesser extent) and could thus be substituted for segment MA to show some function. This assumption seems plausible because segment MA of each subunit shows relatively low sequence homology between different species, but a conserved amphipathic character⁴⁻¹².

Because multiple transmembrane segments may be inserted into the membrane in pairs^{27,28}, it is attractive to assume that segment MA, together with segment M4, is introduced into the membrane. The AChR mutant with the altered α -subunit $\alpha\Delta 394-401$ with a deletion in the region between MA and M4 was not functional, but showed increased apparent binding affinity for the agonist; this suggests some interaction between this region and the agonist binding site. Interestingly, this region of all four AChR subunits contains an excess of negatively-charged amino acid residues over positively-charged residues^{4-8,10-12} (except for the calf muscle AChR γ -subunit⁹). Thus, it is possible that the region between segments MA and M4 is involved in the ligand-dependent gating and the cation selectivity of the AChR channel.

Mutations in the ACh binding region

A disulphide bridge may occur near the ACh binding site of the AChR α -subunit^{1,15}. There are seven cysteine residues in the *T. californica* AChR α -subunit⁴. Two of them (residues 412 and 418), located within segment M4, can be deleted without affecting the apparent binding affinity for the agonist (see mutant $\alpha\Delta 409-420$ described above). Each of the remaining five cysteine residues (128, 142, 192, 193 and 222) was converted into serine by means of oligonucleotide-directed mutagenesis²⁹ (Fig. 3), because this amino acid is similar in structure to cysteine but cannot form a disulphide linkage; these cysteine residues are in the extracellularly located amino-terminal half of the α -subunit (except for residue 222, positioned in segment M1). For the AChR mutants with the altered α -subunit $\alpha 192\text{S}$, $\alpha 193\text{S}$ or $\alpha 222\text{S}$, the contents of the subunit polypeptides were comparable with those of the wild-type AChR, whereas the AChR mutants with $\alpha 128\text{S}$ or $\alpha 142\text{S}$ contained substantially smaller amounts of the α -, γ - and δ -subunits ($\sim 10\%$ of the wild type) (Fig. 4f). Thus, the two latter mutations may affect the conformation of the α -subunit or its assembly with other subunits, thereby reducing the stability of the subunit polypeptides. We have observed previously that, when one of the four subunits is absent, the remaining subunits are found in diminished concentrations¹⁶. The AChR mutants with $\alpha 128\text{S}$ or $\alpha 142\text{S}$ showed hardly detectable or no α -bungarotoxin binding activity and failed to respond to ACh (Table 1), even though the contents of the subunit polypeptides should have been sufficient to elicit a readily measurable response. The AChR mutants with $\alpha 192\text{S}$ or $\alpha 193\text{S}$ had nearly normal α -bungarotoxin binding activity but were not responsive to ACh. Furthermore, the apparent K_D values for the agonist of the mutants with $\alpha 192\text{S}$ or $\alpha 193\text{S}$ were ~ 30 - and ~ 10 -fold higher than that of the wild type, respectively (Fig. 5b). Neither the ACh response nor the agonist binding was impaired in the AChR mutant with $\alpha 222\text{S}$.

Because the mutation of either of two cysteine residues in a disulphide bond is expected to affect the functional properties of the AChR in an analogous manner, our results are consistent with the presence of a disulphide bridge between the cysteine residues 128 and 142 as well as between the cysteine residues 192 and 193. (The disulphide bridge between two adjacent cysteine residues seems unlikely³⁰, but cannot be excluded.) The finding that the mutation of either of residues 128 and 142 leads to a decrease in the concentrations of the AChR subunit polypeptides provides further support for the disulphide linkage between them. Because the mutation of residue 192 or 193 resulted in the complete loss of the ACh sensitivity and in a marked decrease in the apparent binding affinity for the agonist, these residues are probably involved in the binding of ACh and also in signal transduction into the opening of the ionic channel. These cysteine residues are unique to the α -subunit, being conserved in all the fish and mammalian AChR α -subunits analysed^{4,7,11,12}, whereas the cysteine residues 128 and 142 are

conserved in all of the four AChR subunits⁴⁻¹². We speculate that the cysteine residues 192 and 193 of the α -subunit have a specific role in agonist binding and signal transduction, whereas the cysteine residues 128 and 142 of all four subunits, probably forming a disulphide bridge in each subunit, are essential for maintaining the proper conformation of the extracellular region of the AChR molecule; it is possible that the cysteine residues 128 and 142 of the α -subunit are also involved in the agonist binding.

The α -subunit of the *T. californica* AChR contains a single potential site of *N*-glycosidic linkage, the asparagine residue 141, conserved in all four subunits of the AChR⁴⁻¹². This asparagine residue of the α -subunit was converted into aspartic acid (Fig. 3). SDS-polyacrylamide gel electrophoresis of the AChR mutant with the altered α -subunit α 141D revealed that this subunit migrated faster than the wild type (Fig. 4g), suggest-

ing the absence of carbohydrate attachment. A marked decrease in the amount of the δ -subunit ($\sim 10\%$ of the wild type) was observed also, suggesting that the lack of *N*-glycosylation of the α -subunit may affect the assembly of the δ -subunit. This AChR mutant showed hardly detectable α -bungarotoxin binding activity and no ACh response (Table 1), despite the presence of sufficient amounts of the subunit polypeptides. This implies that the *N*-glycosylation at the asparagine residue 141 is essential for the correct conformation of the α -subunit, thus being required for AChR function.

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- Karlin, A. in *Cell Surface Reviews* Vol. 6 (eds Cotman, C. W., Poste, G. & Nicolson, G. L.) 191-260 (North-Holland, Amsterdam, 1980).
- Changeux, J.-P. *Harvey Lect.* 75, 85-254 (1981).
- Conti-Tronconi, B. M. & Raftery, M. A. *Rev. Biochem.* 51, 491-530 (1982).
- Noda, M. *et al. Nature* 299, 793-797 (1982).
- Noda, M. *et al. Nature* 301, 251-255 (1983).
- Noda, M. *et al. Nature* 302, 528-532 (1983).
- Noda, M. *et al. Nature* 305, 818-823 (1983).
- Tanabe, T. *et al. Eur. J. Biochem.* 144, 11-17 (1984).
- Takai, T. *et al. Eur. J. Biochem.* 143, 109-115 (1984).
- Claudio, T., Ballivet, M., Patrick, J. & Heinemann, S. *Proc. natn. Acad. Sci. U.S.A.* 80, 1111-1115 (1983).
- Sumikawa, K. *et al. Nucleic Acids Res.* 10, 5809-5822 (1982).
- Devillers-Thiery, A., Giraudat, J., Bentabollet, M. & Changeux, J.-P. *Proc. natn. Acad. Sci. U.S.A.* 80, 2067-2071 (1983).
- Finer-Moore, J. & Stroud, R. M. *Proc. natn. Acad. Sci. U.S.A.* 81, 155-159 (1984).
- Guy, H. R. *Biophys. J.* 45, 249-261 (1984).
- Karlin, A. *J. gen. Physiol.* 54, 245-264s (1969).
- Mishina, M. *et al. Nature* 307, 604-608 (1984).
- Green, M. R., Maniatis, T. & Melton, D. A. *Cell* 32, 681-694 (1983).
- Krainer, A. R., Maniatis, T., Ruskin, B. & Green, M. R. *Cell* 36, 993-1005 (1984).
- Barnard, E. A., Miledi, R. & Sumikawa, K. *Proc. R. Soc. B215*, 241-246 (1982).
- Miledi, R. *J. Physiol., Lond.* 151, 1-23 (1960).
- Takeuchi, N. *J. Physiol., Lond.* 167, 141-155 (1963).
- Miledi, R. *Proc. R. Soc. B215*, 491-497 (1982).
- Barish, M. E. *J. Physiol., Lond.* 342, 309-325 (1983).
- Haggerty, J. G. & Froehner, S. C. *J. biol. Chem.* 256, 8294-8297 (1981).
- Tzartos, S. J. & Changeux, J.-P. *EMBO J.* 2, 381-387 (1983).
- Kosower, E. M. *Biochem. biophys. Res. Commun.* 111, 1022-1026 (1983).
- Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* 77, 1496-1500 (1980).
- Engelman, D. M. & Steitz, T. A. *Cell* 23, 411-422 (1981).
- Zoller, M. J. & Smith, M. *Meth. Enzym.* 100, 468-500 (1983).
- Dayhoff, M. D. in *Atlas of Protein Sequence and Structure* Vol. 5, 77-268 (National Biomedical Research Foundation, Washington DC, 1976).
- McMaster, G. K. & Carmichael, G. G. *Proc. natn. Acad. Sci. U.S.A.* 74, 4835-4838 (1977).
- King, J. & Laemmli, U. K. *J. molec. Biol.* 62, 465-477 (1971).
- Chamberlain, J. P. *Analyt. Biochem.* 98, 132-135 (1979).
- Kaneda, N., Tanaka, F., Kohno, M., Hayashi, K. & Yagi, K. *Archs Biochem. Biophys.* 218, 376-383 (1982).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* 74, 5463-5467 (1977).
- Messing, J. *Meth. Enzym.* 101, 20-78 (1983).
- Ito, H., Ike, Y., Ikuta, S. & Itakura, K. *Nucleic Acids Res.* 10, 1755-1769 (1982).
- Mishina, M. *et al. EMBO J.* 1, 1533-1538 (1982).

Unique allelic restriction fragments of the human Ha-ras locus in leukocyte and tumour DNAs of cancer patients

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White blood cell DNA from cancer patients and DNA from tumours and tumour-derived cell lines frequently demonstrated allelic restriction fragments of the Harvey ras oncogene locus not found in the unaffected population. The presence of such unusual alleles may be linked to susceptibility to cancer.

SPECIFIC cellular genes^{1,2}, several of which are homologous to the *ras* family of retroviral oncogenes³⁻⁵, have been implicated in the pathogenesis of malignant human tumours. Tumour cell activation of *ras* genes, by single-case mutation of gene-coding sequences⁶⁻⁹, has been detected by transfection of NIH 3T3 mouse cells with DNA preparations from both leukaemias^{10,11} and solid tumours¹²⁻¹⁸.

The human Ha-*ras* gene, homologous to the transforming gene of Harvey murine sarcoma virus³⁻⁵, demonstrates a *Bam*HI restriction fragment length polymorphism (RFLP)¹⁹. DNA sequence data suggest that the basis for this polymorphism is a region of tandemly-repeated nucleotides linked closely to the Ha-*ras* coding sequences²⁰. This structural feature is responsible for the generation of many allelic restriction fragments at the Ha-*ras* locus (see below), a potentially useful circumstance for the genetic analysis of cancer susceptibility. We describe here a population study of Ha-*ras* alleles to determine whether

oncogene RFLPs can be exploited in predicting individual risk and in monitoring the behaviour of tumours.

Characterization of Ha-*ras* polymorphism

We first investigated the basis for Ha-*ras* polymorphism by a Southern blot survey of DNA from white blood cells (WBC) of unrelated Caucasian individuals. DNAs digested with *Bam*HI, blotted and hybridized to radiolabelled Ha-*ras* probe have either a single band or doublet structure of size 6.75-8.7 kilobases (kb). Five of these restriction fragments are shown in Fig. 1a and seem to be allelic. Pedigree analysis (data not shown) and population studies described here support the interpretation that such bands are allelic.

The *Bam*HI fragment containing the EJ/T24 bladder carcinoma oncogene has a region of repetitive DNA ~ 1.5 kb from the 3'-terminus of the coding sequences for Ha-*ras*²⁰. In the molecular clones used for these sequencing studies, the region

Table 1 Distribution of Ha-ras alleles

Allele	Size (kb)	Normal WBC	Patient WBC + tumours	Total alleles	Patient WBC	Tumours	Cell lines
Common							
a1	6.9	158(0.687)	190(0.637)	348(0.659)	102	88	27
a2	7.5	27(0.117)	30(0.101)	57(0.108)	18	12	14
a3	8.0	23(0.100)	23(0.077)	46(0.087)	12	11	2
a4	8.3	13(0.057)	23(0.077)	36(0.068)	13	10	2
Rare							
a4.1	8.4	2(0.0086)	5(0.017)	7(0.013)	2	3	1
a1.1	7.0	2(0.0086)	4(0.013)	6(0.011)	3	1	1
a1.2	7.1	1(0.0043)	5(0.017)	6(0.011)	2	3	0
a5	8.7	3(0.013)	0	3(0.0056)	0	0	0
a2.2	7.8	0	3(0.010)	3(0.0056)	1	2	0
a1.3	7.4	1(0.0043)	1(0.0033)	2(0.0037)	1	0	1
a3.2	8.2	0	2(0.0067)	2(0.0037)	2	0	0
a0.1	6.75	0	2(0.0067)	2(0.0037)	2	0	0
a2.01	7.55	0	2(0.0067)	2(0.0037)	1	1	3
a1.4	7.45	0	2(0.0067)	2(0.0037)	1	1	1
a3.1	8.1	0	1(0.0033)	1(0.0019)	1	0	0
a2.02	7.60	0	1(0.0033)	1(0.0019)	0	1	0
a2.1	7.65	0	1(0.0033)	1(0.0019)	1	0	0
a2.11	7.7	0	1(0.0033)	1(0.0019)	1	0	0
a2.12	7.75	0	1(0.0033)	1(0.0019)	1	0	0
a4.2	8.5	0	1(0.0033)	1(0.0019)	0	1	0
Totals		230	298	528	164	134	52

DNA preparations from WBC and tumours of unrelated Caucasian individuals were purified and processed for Southern blotting as described for Fig. 1. Allelic restriction fragments and estimated size in kb, are listed in order of decreasing frequency of appearance in the total sample (normals + cancer). Numbers in parentheses represent allelic frequencies within each category. The three right hand columns show, separately, the allele data for cancer patient WBC and tumour DNA. Also shown are data from 26 tumour-derived cell lines.

Table 2 Distribution of Ha-ras genotypes

DNA source	Common								Rare	
	a1 a2	a1 a3	a1 a4	a2 a3	a2 a4	a3 a4	a3	a4	a1 a2	a2 a2
WBC (197)										
Normals (115)	21	17	8	2	3	1	1	0	53	0
Patients (82)	15	7	9	2	1	0	1	1	27	0
Tumour tissue (76)										
Leukaemia (55)	7	7	4	0	1	0	0	0	27	0
AML (30)	3	5	1	0	0	0	0	0	14	0
ALL (7)	2	1	0	0	1	0	0	0	2	0
CLL (6)	1	0	0	0	0	0	0	0	4	0
CML (12)	1	1	3	0	0	0	0	0	7	0
Solid tumours (21)	0	2	3	1	1	0	0	0	9	0
Cell lines (26)										
Leukaemia (3)	0	1	0	0	0	0	0	0	2	0
Solid tumour (23)	1	0	1	0	1	0	0	0	9	6
Myelodysplasia (7)	0	0	0	0	0	0	0	0	0	1

DNA preparations from the indicated sources were processed as described for Fig. 1. Genotypes were assigned by the migration of polymorphic Ha-ras fragments. 'Common' genotypes were composed only of alleles a1, a2, a3 and a4. 'Rare' genotypes displayed at least one allele other than a1-a4. The five rare genotypes in the solid-tumour category collectively accounted for six new alleles. Five rare, solid-tumour-cell-line genotypes displayed a total of seven new alleles. Nine paired samples of patient tumour/patient WBC DNA (in which two rare alleles were detected) are included in both the appropriate categories. The alleles and genotypes of these patients are counted only once in Tables 1 and 4, under the patient WBC heading. Abbreviations: AML, acute myelogenous leukaemia; ALL, acute lymphocytic leukaemia; CML, chronic myelogenous leukaemia; CLL, chronic lymphocytic leukaemia.

consists of variable tandem repetition (VTR) of a 28-base-pair (bp) consensus sequence. It has therefore been suggested that the *Bam*HI RFLP arises by changes in the number of repeat units²⁰, supported here by our present finding that the difference in *Bam*HI fragment sizes (Fig. 1b) is accounted for always by the difference in *Msp*I/*Hpa*II fragment sizes (Fig. 1c). (*Msp*I/*Hpa*II sites closely flank the VTR region; Fig. 1d.) Occasional and irreproducible discrepancies of 0.1 kb have occurred. In addition, the RFLP is demonstrated more easily with *Msp*I/*Hpa*II than *Bam*HI, thus allowing for more sensitive

detection of fragments with altered migration (compare Fig. 1b and c).

Distribution of Ha-ras alleles and genotypes

We next determined whether unique Ha-ras alleles could be detected in the genomes of cancer patients and if the distribution of genotypes among these patients was altered. Because the greatest resolution of the Ha-ras RFLP was obtained with *Msp*I/*Hpa*II, we used this endonuclease combination for screening. Combined use also reduced the effect of DNA methyl-

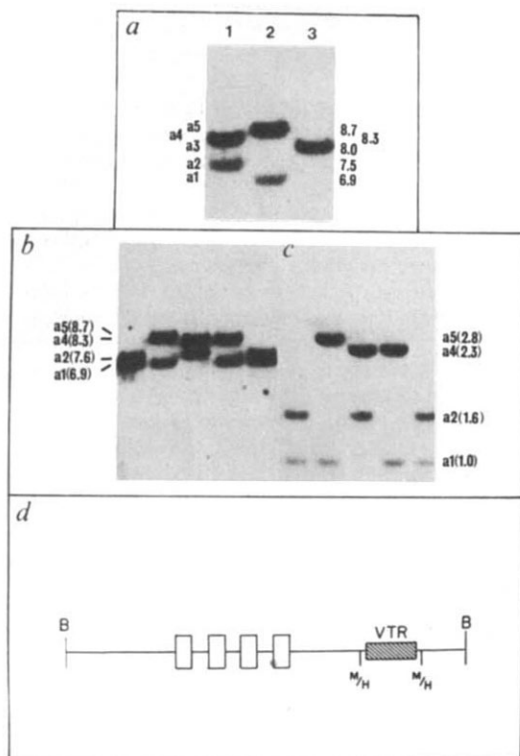


Fig. 1 Ha-*ras* alleles in normal white blood cell DNA. *a*, Three WBC DNAs digested with *Bam*HI and fractionated on a 0.7% agarose gel before Southern blotting. *b*, Five WBC DNAs digested with *Bam*HI and fractionated on a 0.7% gel. *c*, The same five DNAs digested with *Msp*I and *Hpa*II, then electrophoresed on a 1.4% agarose gel. *d*, Schematic representation of the *Bam*HI (B) fragment containing the human Ha-*ras* gene. Open boxes, exons; hatched box, region of VTR. Many more *Msp*I/*Hpa*II (M/H) sites occur near and within the Ha-*ras* gene, but the corresponding fragments are too small to be visualized on the 1.4% gels used here. Peripheral white blood cells of normal individuals were prepared by dextran sedimentation. DNA was purified¹³ and 5 µg each sample digested to completion with the indicated restriction enzyme. Following electrophoresis on agarose gels, DNA was transferred to a nitrocellulose filter by the method of Southern³⁰. The *Bam*HI fragment of plasmid pEC (ref. 29) containing the Ha-*ras* gene was purified and nick-translated³¹ to a specific activity of 1.5×10^8 c.p.m. µg⁻¹. Filter hybridization, wash and autoradiography were performed as described⁵. Fragment sizes were determined by co-electrophoresis with *Hind*III-digested λ DNA.

ation on digestion. *Bam*HI was used in independent digestions of samples yielding new alleles to prove that incomplete digestion and *Msp*I-site polymorphism were not sources of unusual restriction fragments. Data from 197 WBC and 76 tumour-tissue DNA samples of unrelated individuals, as well as from 26 tumour-derived cell line DNA samples, are summarized in Tables 1 and 2. 'Normal' WBC DNA was obtained from individuals without cancer or family history of cancer in first-degree relatives, including patients from the General Medicine clinic of the hospital, medical centre staff and cancer patient spouses. 'Cancer' genotypes, both WBC and tumour, were from patients chosen consecutively from our haematology-oncology clinics. There was no discernible difference in the ethnic composition of the two groups.

Twenty distinct allelic restriction fragments were noted in the 528 alleles sampled (Table 1). Examination of the allelic frequencies showed that Ha-*ras* alleles could be divided into two groups, the first ('Common') consisting of four alleles occurring with frequencies of 7, 9, 10 and 66% (~93% of the total) and the second, ('Rare') composed of the remaining 16 alleles of individual frequency 0.2–1.3%. Of the rare alleles, four (a1.1, a1.2, a1.3 and a4.1) occurred in both normal and

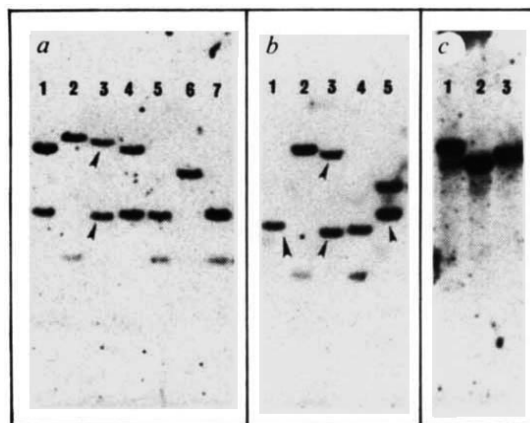


Fig. 2 Ha-*ras* alleles in tumour DNA. Five µg tumour or normal marker DNA were digested with *Msp*I and *Hpa*II (*a*, *b*) or with *Bam*HI (*c*). Electrophoresis of *Msp*I/*Hpa*II digests was on 1.4% agarose gels, *Bam*HI digests on 0.7% gels. Blotting and hybridization as described in Fig. 1. *a*, Lane 1, marker DNA containing a2 and a4 alleles; lane 2, marker DNA containing a1 and a5 alleles; lane 3, ovarian carcinoma DNA 1; lane 4, ovarian carcinoma DNA 2; lane 5, marker DNA containing a1 and a2 alleles; lane 6, oesophageal carcinoma DNA with a3 allele; lane 7, acute lymphocytic leukaemia DNA. *b*, Lane 1, lung carcinoma DNA; lane 2, marker DNA containing a1 and a5 alleles; lane 3, ovarian carcinoma DNA number 1; lane 4, marker DNA containing a1 and a2 alleles; lane 5, WBC DNA from patient with familial melanoma. *c*, Lane 1, oesophageal carcinoma DNA; lane 2, marker DNA containing a1 allele; lane 3, lung carcinoma DNA.

cancer genotypes, one (a5) was detected only in normals and the remaining 11 occurred only in cancer genomes. Thus, the Ha-*ras* locus showed more heterogeneity in cancer than normal genomes. The same relative distribution of alleles into the two groups was found in both patient WBC and tumour DNAs (Table 1).

Rare Ha-*ras* restriction fragments in WBC and tumour DNA of cancer patients (Fig. 2) show sometimes that two new alleles appear in the same sample (for example, the ovarian carcinoma of Fig. 2*a*, lane 3; *b*, lane 3). Three affected members of a familial melanoma kindred share the same rare allele (Fig. 2*b*, lane 5). (In each case *Bam*HI digestion confirmed the result.) Among solid tumours, unusual fragments were present in a melanoma, a lymphoma and carcinomas of ovary and lung. All forms of leukaemia (except chronic myelogenous leukaemia) demonstrated rare alleles. We used five DNA samples with uncommon restriction fragments, including one WBC DNA from the melanoma kindred, to transfect NIH 3T3 cells by previously described methods¹⁴; in no case was transforming activity detected.

Although the rare WBC allele of Fig. 2, lane 5 was from a patient with a familial tumour syndrome, such Ha-*ras* alleles were also detected in WBC DNA of patients with sporadic tumours (Fig. 3, lane 1W). Because the new alleles were detected by *Msp*I/*Hpa*II, we suggest that the Ha-*ras* polymorphisms of cancer patient WBC and tumour DNA are also generated in the VTR region. Digestion with *Bam*HI supports this interpretation in all examples except a1.4 and a2.01. These alleles were too like the a2 allele to be resolved with *Bam*HI, so we could not exclude the possibility that a1.4 and a2.01 arose by *Msp*I-site polymorphism.

The distribution of Ha-*ras* genotypes is shown in Table 2. An examination of common genotypes among normal WBC DNAs supports our earlier statement that the *Msp*I/*Hpa*II polymorphic fragments show allelism. For example, the Hardy-Weinberg relation predicts that the number of expected a1/a2 genotypes in the sample of 115 should be $(115 \times 2 \times 0.687 \times 0.117)$, or 18.5; 21 were observed. Similarly, the observed (expected) values for genotypes a1/a3, a1/a4 and a1/a1 were 17 (15.8), 8 (9) and 53 (54.2), respectively. Other common genotypes gave

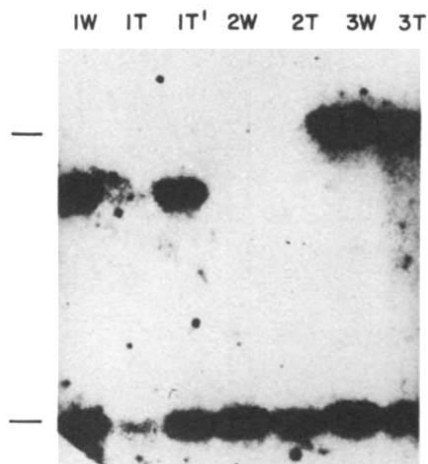


Fig. 3 Ha-*ras* alleles in paired DNAs from patients' WBC and tumours. DNA from WBC (W) and tumours (T) of patients 1-3 were purified, digested with *MspI/HpaII* and processed for hybridization to Ha-*ras* probe as described in Fig. 1. Bars mark the migration of common alleles a1 and a4. Two distinct metastases (IT and IT') were the DNA sources from patient 1 (melanoma). Primary tumour DNA was obtained from patient 2 (breast carcinoma). A skin metastasis was the DNA source from patient 3 (melanoma).

similar results, leading us to conclude that the segregation of common polymorphic fragments in the normal population is characteristic of independent alleles.

When the genotype distribution of normal WBC DNA was compared with that of cancer WBC and tumour DNA, several patterns emerged. Common genotypes occurred in the same relative proportion in each group of DNAs. (The under-representation of a1/a3 in tumour DNAs, for example, was not statistically significant.) Genotypes containing rare alleles, however, particularly those occurring only once or twice, were more frequent in patient WBC, tumour and tumour-derived cell-line categories. For example, 23% of cancer patient WBC genotypes showed very rare alleles, compared with 9% in the cancer-free population; no single-occurrence allele was found in normal genomes. Six of seven patients with myelodysplasia,

a disorder characterized by dysplastic changes in haematopoietic cells with frequent progression to acute leukaemia, displayed rare alleles.

The individual cases and diagnoses in which rare alleles were detected (Table 3) show that the preleukaemic state of myelodysplasia is associated with several of the same rare alleles found in acute myelogenous leukaemia (AML). The allele a2.2, for example, occurs in one case of AML preceded by myelodysplasia, in unrelated cases of AML and myelodysplasia and in a case of essential thrombocythaemia (unexplained increase in platelet production). Other associations between particular alleles and a given class of tumours may be discovered when a larger number of patient DNA samples has been screened.

The over-representation of rare alleles in cancer genotypes was statistically significant ($P < 0.01$) by χ^2 analysis (Table 4). As the overall distribution of common genotypes was the same in normal and cancer populations, no common allele was excluded preferentially in the cancer population to accommodate the increased number of rare alleles.

Ha-*ras* locus instability

Several other properties of the Ha-*ras* locus in tumours and tumour-derived cell lines were noted. First, none of the 98 tumour and tumour-derived cell line DNAs showed any significant amplification of Ha-*ras*. Unlike *myc* and *Ki-ras*, loci where gene amplification can occur^{21,22}, Ha-*ras* demonstrated a remarkably well-conserved copy number, but the locus was involved frequently in loss of one allele. This happened occasionally in tumours and often in cell lines. We could detect subtle changes in relative copy number of Ha-*ras* alleles because of the characteristic changes in band intensity associated with fragment size; as larger fragments contained more tandem repeats, the hybridization intensity was proportional to fragment size (for example, Fig. 2a, lanes 1 and 2). Tumours often contained altered allelic ratios (Fig. 2a; compare marker ratios in lanes 1, 2 and 5 with tumour DNAs in lanes 3, 4 and 7). Occasionally, nearly complete loss of an allele occurred. For example, the carcinomas of Fig. 2a, lane 6 and b, lane 1, appear homozygous. When a *Bam*HI digest of these DNAs was hybridized to probe and the autoradiogram deliberately overexposed, however, a minor component of the a1 allele could be demonstrated (Fig. 2c, lanes 1 and 3). Finally, cell line DNAs showed Ha-*ras* homozygosity in excess of predictions from allelic

Table 3 Tumour types associated with rare Ha-*ras* alleles

	AML	Mdys.	Lymph.	Brst.	Sarc.	Ovar.	Colon	Mela.	Miscellaneous
a1.1	T	W		W*	W + T				
a1.2	T*	W	T						Basal cell (W) (1)
a2.2	T*	W*							Thromb'cyth. (W) (1)
a1.3									Mult. myel. (W) (2)
a3.2			W				W		
a0.1	W†			W					
a2.01	T	W							Glioblastoma (W) (1)
a1.4				W		T‡			
a3.1								W + T	
a2.02									Lung (T) (5)
a2.1								W	
a2.11							W		
a2.12		W	W						
a4.2	T								
a4.1					W	T‡			ALL (T) (7)/CLL (T) (6)/VHL (W) (1)

Each row shows the rare alleles in a given tumour type; each column shows tumour types associated with a given rare allele. The final column contains various tumours in which only one rare allele was detected. The numbers in parentheses represent the total number of patients screened. Two cases of AML preceded by myelodysplasia are listed under both categories (alleles a1.2 and a2.2). The results are derived from 30 cases of AML, 7 of myelodysplasia, 7 lymphomas, 13 breast carcinomas, 3 sarcomas, 5 ovarian carcinomas, 7 colon carcinomas and 3 melanomas. Tumour types tested, but not yet demonstrating rare alleles (for example, gastric carcinoma), are not listed. Abbreviations: AML, acute myelogenous leukaemia; Mdys., myelodysplasia; Lymph., lymphoma; Brst., breast; Sarc., sarcoma; Ovar., ovarian; Mela., melanoma; ALL, acute lymphocyte leukaemia; CLL, chronic lymphocytic leukaemia; Thromb'cyth., essential thrombocythaemia; Mult. myel., multiple myeloma; VHL, Von Hippel Lindau; T, allele detected in tumour DNA; W, allele detected in WBC DNA; W + T, paired WBC and tumour samples.

* Two cases; † remission WBC; ‡ same tumour.

Table 4 Comparison of rare alleles in normal and cancer DNA

DNA source	Alleles		Probability
	Common	Rare	
Normal WBC	221	9	—
Tumours + cancer WBC	266	32	<0.01
Cancer WBC	145	19	<0.01
Tumours	121	13	0.03

The column designations 'Common' and 'Rare' refer to the groups described in Table 1. χ^2 analysis (1 d.f.) was performed to compare the distribution of genotypes in normal WBC DNA versus tumour + cancer WBC DNAs. Also compared were normal WBC DNAs versus cancer WBC DNAs alone and versus tumour DNA alone. Genotype data from myelodysplasia and from cell lines were not included.

frequencies (Table 2). There was a significant increase of a2/a2 genotypes. These findings all indicate that, whereas the Ha-ras locus of tumours and tumour-derived cell lines was quite unstable in the maintenance of heterozygosity, some constraints operated to keep the locus close to diploid copy number.

Origin of rare alleles

As Ha-ras allelic variation was associated with tandemly-repeated DNA and as such DNA may provide an unstable site where recombination or amplification could occur, we speculated that unique alleles are generated within tumours. This possibility proved unlikely, however, for three reasons. First, as reported here, unique alleles appeared as frequently in patient WBC DNA as in tumour DNA. Second, we observed no discordance between patient WBC and patient tumour genotypes in nine paired samples (see Fig. 3). None of the common alleles present in WBC DNA were replaced by rare alleles in the tumour DNAs. In addition, two of the nine cases demonstrated rare alleles present in both tumour and WBC genotypes. Finally, in cases where the patient's germline alleles were not available for comparison with unique tumour alleles, restriction mapping demonstrated that normal restriction sites within 10 kb upstream and downstream of the VTR were preserved (data not shown). Thus, it was unlikely that a major rearrangement had occurred by recombination within the VTR region. We conclude that generation of rare restriction fragments within tumours, although it may occur sporadically, is not the predominant mode by which these alleles have arisen.

Another possibility is that these variants might arise *in utero*; such an appearance of a tumour susceptibility marker has been documented in one patient with retinoblastoma²³. When we examined first-degree relatives of patients with rare alleles in WBC DNA (5 of the 19 cases in Table 2), at least one relative also possessed the allele. For example, the fragment denoted by the arrow in Fig. 2b, lane 5, was shared by several members of the melanoma kindred.

These observations, although not excluding the generation of unusual alleles by events in tumours or *in utero*, support the hypothesis that they were transmitted most frequently as mendelian alleles of the Ha-ras locus.

Discussion

Our survey of allelic restriction fragments of the human Ha-ras locus demonstrates the frequent appearance of unique alleles in cancer patients' WBC and tumour DNA not detected in individuals without cancer or a family history of cancer. These alleles are inherited in a mendelian fashion, rather than arising in tumours or *in utero*. The allelic fragments are generated apparently in the same manner as Ha-ras alleles in the cancer-free population; that is, by variation in the number of tandem repeats 3' to the Ha-ras coding sequences. The enrichment of very rare Ha-ras allelic restriction fragments in cancer patient

DNA implies that the RFLP at this locus will be useful in the assessment of individual risk of tumour development.

We also observed Ha-ras locus instability in tumours and tumour-derived cell lines, commonly manifested as loss of one allele. As allelic loss may be a marker for events leading to the expression and consequent selection of recessive phenotypes in many types of tumours, the monitoring of locus instability may prove an important tool for studying tumour progression, as demonstrated for retinoblastoma²⁴ and probably for Wilm's tumour²⁵⁻²⁸. It will be useful to determine whether genes associated with VTRs are particularly unstable in tumours. As tandem repeat markers other than Ha-ras are now available (M.C. and T.G.K., in preparation), a more comprehensive analysis of such loci in tumours may soon be possible.

Why should the variation of tandemly-repeated nucleotides affect risk? Such influence might be mediated via the Ha-ras gene itself. The unusual VTRs reported here are perhaps in linkage disequilibrium with Ha-ras mutations which, although not sufficiently potent to be detected by transfection, may still contribute to oncogenesis. Linkage disequilibrium with unrelated genes near Ha-ras is possible also. These two hypotheses, however, require that the generation of a rare VTR occurs during the process which results in the appearance of the 'high-risk' gene. Such events, leading to marked variation in the tandem repeats near Ha-ras, may reflect a source of genomic instability in those destined to develop cancer. In this regard, it is interesting that more than one unusual Ha-ras allele almost always appears in kindreds with very prominent histories of cancer (N.A.D., D.R.P. and T.G.K., unpublished observations). Finally, the VTRs may themselves be enhancer elements. Thus, the level of expression of Ha-ras, which can determine its propensity for cell transformation as effectively as mutation²⁹, may be altered.

Functional analysis of molecular clones of unusual alleles, particularly those present in myelodysplasia, and continuing population analysis of Ha-ras and other VTR-linked loci, may clarify many of these issues.

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Note added in proof: The transforming activity of EJ-ras subclones lacking the VTR region is 5- to 10-fold lower than that of the original clone (M.C., R. Geller and T.G.K., unpublished results). The expression of the p21 gene product is also reduced³².

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- Cooper, G. M. *Science* **218**, 801-806 (1982).
- Bishop, J. M. *Cell* **32**, 1018-1020 (1983).
- Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. H. *Nature* **297**, 474-478 (1982).
- Santos, E. *et al. Nature* **298**, 343-347 (1982).
- Der, C. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3637-3640 (1982).
- Tabin, C. J. *et al. Nature* **300**, 143-149 (1982).
- Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. *Nature* **300**, 149-152 (1982).
- Shimizu, K. *et al. Nature* **304**, 497-500 (1983).
- Capon, D. J. *et al. Nature* **304**, 507-513 (1983).
- Murray, M. J. *et al. Cell* **33**, 749-757 (1983).
- Eva, A., Tronick, S. R., Gol, R. A., Pierce, J. H. & Aaronson, S. A. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4926-4930 (1983).
- Shih, C., Padhy, L. C., Murray, M. & Weinberg, R. A. *Nature* **290**, 261-264 (1981).
- Perucho, M. *et al. Cell* **27**, 467-476 (1981).
- Krontiris, T. G. & Coper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1181-1184 (1981).
- Murray, M. J. *et al. Cell* **25**, 355-361 (1981).
- Pulciani, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 2845-2849 (1982).
- Marshall, C. J., Hall, A. & Weiss, R. A. *Nature* **299**, 171-173 (1982).

18. Pulciani, S. *et al.* *Nature* **300**, 539–542 (1982).
19. Goldfarb, M., Shimizu, K., Peruchio, M. & Wigler, M. *Nature* **296**, 404–409 (1982).
20. Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. & Goeddel, D. V. *Nature* **302**, 33–37 (1983).
21. Collins, S. J. & Groudine, M. *Nature* **298**, 679–681 (1982).
22. Schwab, M., Alitudo, K., Varnus, H. E., Bishop, J. M. & George, D. *Nature* **303**, 497–501 (1983).
23. Benedict, W. F. *et al.* *Science* **219**, 973–975 (1983).
24. Cavenee, W. J. *et al.* *Nature* **305**, 779–784 (1983).
25. Koufos, A. *et al.* *Nature* **309**, 170–172 (1984).
26. Orkin, S. H., Goldman, D. S. & Sallon, S. E. *Nature* **309**, 172–174 (1984).
27. Reeve, A. E. *et al.* *Nature* **309**, 174–176 (1984).
28. Fearon, E. R., Vogelstein, B. & Feinberg, A. P. *Nature* **309**, 176–178 (1984).
29. Chang, E. H., Furth, M. E., Scolnick, E. M. & Lowry, D. R. *Nature* **297**, 479–483 (1982).
30. Southern, E. M. *J. molec. Biol.* **98**, 503–513 (1975).
31. Maniatis, T., Jeffrey, A. & Kleid, D. G. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1184–1188 (1975).
32. Seeburg, P. H. *et al.* *Nature* **312**, 71–75 (1984).

LETTERS TO NATURE

A second measurement of a pulsar braking index

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The braking index, n , of a pulsar, which describes the dependence of the braking torque on rotation frequency, is a fundamental parameter of pulsar electrodynamics. Simple theoretical arguments, based on the assumption of a constant dipolar magnetic field, predict $n = 3$, whereas the only previously measured value (for the Crab pulsar) is 2.515 ± 0.005 . Here we present radio timing observations of the X-ray and radio pulsar, PSR1509–58, made over an approximately 2-year interval, which yield a second significant measurement of $n = 2.83 \pm 0.03$, more than that for the Crab pulsar, but less than the canonical value of 3. Despite the large period first derivative of PSR1509–58, there is no evidence for glitches or random irregularities in the period.

The braking index of a pulsar, is defined by the torque equation, $\dot{\nu} = -K\nu^n$, where ν is the pulsar rotation frequency, $\dot{\nu}$ is its first derivative with respect to time and K is a constant. For a dipolar magnetic field, braking of the star's rotation by radiation reaction^{1–3}, or by ejection of charged particles⁴, both give $n = 3$. From this definition, $n = \nu\ddot{\nu}/\dot{\nu}^2$. The pulsar frequency and its first derivative are relatively easy to measure, so the determination of the braking index rests on a measurement of the second time derivative of the frequency (or period). To date, there has been only one significant determination⁵, that for the Crab pulsar for which $n = 2.515 \pm 0.005$, as for other pulsars the secular second-derivative term has been masked by the presence of glitches and/or random irregularities in period^{6–9}. PSR1509–58, however, discovered first at X-ray wavelengths¹⁰ and detected subsequently as a radio pulsar¹¹, has a relatively short period, 0.150 s, and a very large period-derivative, $\sim 1.5 \times 10^{-12} \text{ s s}^{-1}$, more than three times that of the Crab pulsar, previously the largest known; it is therefore a good candidate for measurement of the braking index as its second derivative, resulting from the secular slow-down, should be relatively large.

Observations were made at the Molonglo Radio Observatory using the Molonglo synthesis telescope (MOST) in total-power mode. MOST operates at 843 MHz and has a beam of dimensions at transit $41 \text{ arcs} \times 2^\circ$ which can track sources to low elevations¹². The effective antenna area is $9,000 \text{ m}^2$ corresponding $\sim 0.3 \text{ Jy K}^{-1}$, and the intermediate-frequency bandwidth is $\sim 3 \text{ MHz}$. Before May 1983, the system temperature on cold sky was $\sim 250 \text{ K}$, but then installation of improved preamplifiers reduced the system temperature to $\sim 110 \text{ K}$. The detected output was sampled at 2-ms intervals and, using an off-line computer, integrated synchronously with the apparent pulsar period; the resultant pulse profile was cross-correlated with a reference profile to give a pulse arrival time.

PSR1509–58 was observed, together with a reference pulsar, PSR1451–68, a total of 109 times between 1982 June 6 and 1984 April 8. Each observation of PSR1509–58 was of 4,000-s duration and typically yielded a pulse profile with a peak/r.m.s.-

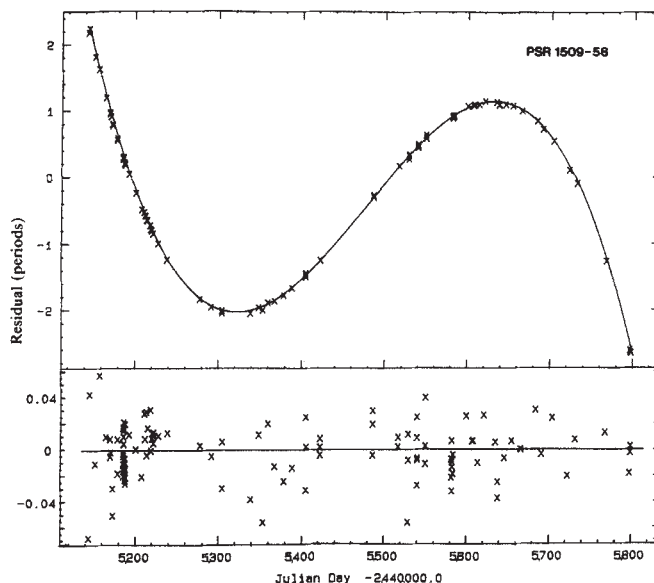


Fig. 1 Residuals (observed – predicted) from fits to pulse-arrival-time data for PSR1509–58. For the upper panel, the parameters fitted are pulsar position, phase zero, frequency and frequency derivative. The solid line shows the fit when a cubic term representing the frequency-2nd-derivative is included. Residuals from the latter fit are shown enlarged in the lower panel.

noise ratio of 10. The arrival times from the cross-correlation analysis were corrected to the Solar System barycentre using the MIT ephemeris and the pulsar position and period parameters calculated using standard techniques¹³.

Parameters derived from fitting the position, phase zero, pulse frequency, frequency derivative and frequency-second derivative are given in Table 1, where errors quoted are twice the formal standard deviation (the error in pulse frequency is in the last digit quoted). The position does not include the E-terms of aberration, $\Delta\alpha = -0.026 \text{ s}$ and $\Delta\delta = +0.22''$, which must be added to the quoted position to convert it to the FK4 system. The quoted first derivative, $\dot{\nu}$, is equivalent to a period derivative of $(1.540189 \pm 0.000006) \times 10^{-12} \text{ s s}^{-1}$, consistent with, but more accurate than, the values based on X-ray measurements¹⁴. Figure 1 shows the residuals from a fit omitting the second-derivative term (upper) and the residuals from the final fit (lower). The cubic phase term representing the second derivative is clearly evident in the upper panel and the lower panel shows that the residuals from the final fit can be completely accounted for by measurement error. There is no evidence for any reduction in the amplitude of the residuals following the improvement in the preamplifiers (that is, after Julian day 2,445,460) indicating that the residuals are dominated by effects other than noise. Across the 3-MHz bandwidth the interstellar dispersive delay changes by $\sim 10 \text{ ms}$. Thus, relative enhancement of the pulsar signal in a portion of the received bandpass could account for most of the observed 2.8-ms-r.m.s. scatter and, as a bandwidth of $\sim 1 \text{ MHz}$ for interstellar scintillations is not outside the range of expected values¹⁵, we suggest that this effect accounts for the bulk of the observed residuals.

The second-derivative term is apparently very significant, being ~ 200 times the estimated error, but experience with other pulsars has shown that random irregularities in the pulsar period often produce a strong cubic component in the phase residuals (see, for example, ref. 9). Indeed, it is characteristic of such fits that they fail to predict the pulse phase for any reasonable length of time (for example, a substantial fraction of the interval fitted) and that the magnitude of the second-derivative parameter is dependent on the length of the data interval fitted. On both of these counts, PSR1509–58 performs well; a fit to the data up to end of 1983 gave a frequency-second derivative of $1.960 \times 10^{-21} \text{ s}^{-3}$, only 1% different from the value in Table 1, and this fit predicts the phase to the end of the present data set to better than 0.1 periods.

As a diagnostic for the effects of random processes, Cordes and Helfand⁶ suggest a parameter $r_{32} = \sigma_R(3, T)/\sigma_R(2, T)$, where $\sigma_R(n, T)$ is the r.m.s. residual due to random processes for a fit of order n to arrival-time data spanning an interval T . If we make the conservative assumption that measurement error and random processes contribute equally to the observed variance, then, for PSR1509–58, $\sigma_R(3, 650 \text{ day}) \sim 2 \text{ ms}$, which, with the r.m.s. residual for the second-order fit (upper panel of Fig. 1) of $\sim 160 \text{ ms}$, gives an apparent value for r_{32} of 0.01. For random processes $r_{32} \sim 0.5$; a value as low as 0.01 is inconsistent with phase noise and frequency noise and is unlikely to be obtained with slowing-down noise⁶.

We therefore conclude that the derived second-derivative term is not dominated by random processes, but represents the secular effect expected on the basis of the torque law. The value derived for the braking index is $n = 2.83 \pm 0.03$, where the estimated error has again been doubled to allow for possible contamination of the second derivative by random processes.

In summary, the good fit of the standard timing model to the observed pulse arrival times rules out any remaining possibility that PSR1509–58 is a member of a binary system of the type which would allow the X-ray emission to be powered by accretion; PSR1509–58 is therefore the second example (after the Crab pulsar) of an isolated pulsar emitting pulsed radiation at X-ray wavelengths.

The derived FK4 position, $\alpha = 15 \text{ h } 09 \text{ min } 59.03 \text{ s}$, $\delta = -58^\circ 56' 57.9''$, is $3.6''$ east and $0.9''$ south of the X-ray position given by Seward and Harnden¹⁰ and within the error circle (5-arc s radius) quoted by them.

It is significant that these observations have shown no evidence for either glitches or more continuous random irregularities in the pulsar period. Models involving unpinning and subsequent recoupling of vortex lines in the superfluid region of neutron star interiors¹⁶ seem to provide the most satisfactory explanation of the large glitches observed in the Vela and other pulsars. Alpar and Ho¹⁷ suggest that the time between glitches is given by $t_g = \delta\Omega/|\dot{\Omega}|$, where $|\dot{\Omega}| = 2\pi|\dot{\nu}|$ is the slowing down rate and $\delta\Omega$ is a critical frequency increment. From the observed glitches, values of $\delta\Omega$ in the range 10^{-3} – $10^{-2} \text{ rad s}^{-1}$ are obtained. Because of the large frequency derivative, predicted glitch intervals for PSR1509–58 are very short, of 30–300 days, but no major glitch has occurred in the ~ 650 -day interval observed so far. Continuing observations will put stronger limits on the actual glitch frequency.

Pulsars with large period derivatives tend to have stronger random irregularities in their period⁶. An 'activity parameter', $A = \log(\sigma_R(2, T)/\sigma_R(2, T)_{\text{Crab}})$, can be defined⁶ as a quantitative estimate of the level of these timing irregularities. For the Crab pulsar, $\sigma_R(2, 1,600 \text{ day}) \approx 12 \text{ ms}$; as the Crab timing irregularities have the character of frequency noise⁵, $\langle \sigma_R(n, T) \rangle \propto T^{3/2}$, and so $\sigma_R(2, 650 \text{ day})_{\text{Crab}} \approx 3 \text{ ms}$. For random processes $r_{32} \approx 0.5$, so we take $\sigma_R(2, 650 \text{ day})$ to be $< 4 \text{ ms}$ for PSR1509–58. Therefore PSR1509–58 has timing irregularities comparable with, or less than, those in the Crab pulsar, that is, $A < 0.1$, whereas stronger irregularities would be expected on the basis of the large period derivative. As the observed residuals can be fully accounted for by measurement error, it is clearly desirable to reduce the size of this error.

Table 1 Parameters of PSR1509–58 from pulse-timing analysis

RA (1950.0)	$\alpha = 15 \text{ h } 09 \text{ min } 59.06 \text{ s} \pm 0.07 \text{ s}$
Dec. (1950.0)	$\delta = -58^\circ 56' 58.1'' \pm 0.7''$
Frequency	$\nu = 6.656424796 \pm 3 \text{ s}^{-1}$
First derivative	$\dot{\nu} = (-6.82427 \pm 0.00003) \times 10^{-11} \text{ s}^{-2}$
Second derivative	$\ddot{\nu} = (1.982 \pm 0.009) \times 10^{-21} \text{ s}^{-3}$
Epoch (Julian day)	$= 2,445,144.7437$
R.m.s. residual	$= 2.8 \text{ ms}$

The measured braking index for PSR1509–58, 2.83 ± 0.03 , lies between the theoretically-predicted value, 3.0, for a dipole field, and the only other measured value, 2.515, for the Crab pulsar⁵. Many factors can influence the braking index of a pulsar; the most probable reason for a braking index of < 3 , is radial deformation of the magnetic-field lines, for example, by an outflowing plasma^{13,18}. In the limiting case of a stellar wind¹⁹, $n = 1$. If plasma contributes significantly to the braking of pulsar rotation, the radius of the Alfvén cylinder must be comparable to, or less than, that of the light cylinder. The Alfvén cylinder radius is defined by $B^2/8\pi \approx \rho r_A^2 \Omega^2$, where $\Omega = 2\pi\nu$ and B and ρ are respectively, the magnetic-flux density and particle-mass density at $r = r_A$. Hence, $r_A \approx \mu^{2/5}/(M\Omega)^{1/5}$, where $\mu \approx Br^3$ is the magnetic moment of the neutron star and $\dot{M} \approx 4\pi\rho\Omega r_A^2$ is the total mass-loss rate. The braking torque resulting from tangential acceleration of the plasma is $-N \approx M\Omega r_A^2 \approx \dot{M}^{3/5} \mu^{4/5} \Omega^{3/5}$. The effective braking index is therefore determined by the Ω -dependence of $\dot{M}$, and requiring that $r_A < c/\Omega$, the light cylinder radius, gives $\dot{M} > \mu^2 \Omega^4/c^5$. The condition of equality gives $n = 3$, whereas $n < 3$ if $\dot{M}$ varies with Ω more slowly than Ω^4 . The observation that n is < 3 is strong evidence that a large fraction of the angular momentum and energy loss from the pulsar is in the form of particles, although it is possible that vacuum conditions apply in some portions of the magnetosphere with other portions being plasma dominated. The same applies of course to the Crab pulsar and we note that both pulsars emit X-ray pulses and are surrounded by nonthermal X-ray nebulae²⁰.

For a constant braking index, the age of a pulsar, t , is given by

$$t = -\frac{\nu}{(n-1)\dot{\nu}} \left[1 - \left(\frac{\nu}{\nu_i} \right)^{n-1} \right] \quad (1)$$

where ν_i is the pulse frequency at $t = 0$. For $\nu_i \gg \nu$, $t = -\nu/(n-1)\dot{\nu} = 1,690 \text{ yr}$ for PSR1509–58, but, if the braking index increases with time, the pulsar age may be (slightly) greater than this. On the other hand, if ν_i is not very large compared with the present ν , then the age will be less. In conclusion the small age of PSR1509–58 is confirmed by the present observations and the question of its association with the possibly-much-older supernova remnant G320.4–1.2 or MSH15–52 remains somewhat controversial^{21–23}.

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1. Pacini, F. *Nature* **216**, 567–568 (1967).
2. Pacini, F. *Nature* **219**, 145–146 (1968).
3. Ostriker, J. P. & Gunn, J. E. *Astrophys. J.* **157**, 1395–1417 (1969).
4. Goldreich, P. & Julian, W. H. *Astrophys. J.* **157**, 869–880 (1969).
5. Groth, E. J. *Astrophys. J. Suppl.* **29**, 431–441 (1975).
6. Cordes, J. M. & Helfand, D. J. *Astrophys. J.* **239**, 640–650 (1980).
7. Downs, G. S. *Astrophys. J.* **249**, 687–697 (1981).
8. Gullahorn, G. E. & Rankin, J. M. *Astrophys. J.* **260**, 520–537 (1982).
9. Manchester, R. N., Newton, L. M., Hamilton, P. A. & Goss, W. M. *Mon. Not. R. astr. Soc.* **202**, 269–285 (1983).
10. Seward, F. D. & Harnden, F. R. *Astrophys. J. Lett.* **256**, L45–L47 (1982).
11. Manchester, R. N., Tuohy, I. R. & D'Amico, N. *Astrophys. J. Lett.* **262**, L31–L33 (1982).
12. Mills, B. Y. *Proc. Astr. Soc. Aust.* **4**, 156–159 (1981).
13. Manchester, R. N. & Taylor, J. H. *Pulsars* (Freeman, San Francisco, 1977).
14. Weisskopf, M. C. et al. *Astrophys. J.* **267**, 711–712 (1983).

15. Rickett, B. J. *Astr. Astrophys.* **15**, 479–504 (1977).
16. Alpar, M. A., Anderson, P. W., Pines, D. & Shaham, J. *Astrophys. J.* **276**, 325–334 (1984).
17. Alpar, M. A. & Cheng, H. *Mon. Not. R. astr. Soc.* **204**, 655–667 (1983).
18. Roberts, D. H. & Sturrock, P. A. *Astrophys. J.* **181**, 161–180 (1973).
19. Michel, F. C. *Astrophys. J.* **158**, 727–738 (1969).
20. Seward, F. *IAU Symp.* **101**, 405–416 (1983).
21. Manchester, R. N. & Durdin, J. M. *IAU Symp.* **101**, 421–427 (1983).
22. Van den Bergh, S. & Kamper, K. W. *Astrophys. J. Lett.* **280**, L51–L54 (1984).
23. Seward, F. D., Harnden, F. R., Murdin, P. G. & Clark, D. H. *Astrophys. J.* **267**, 698–710 (1983).

The peculiar X-ray and radio star AS431

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During a systematic survey of X-ray flux-limited late-type stars^{1,2}, we have rediscovered a highly reddened emission-line star, previously listed³ as AS431, which sheds light on whether both radio and X-ray emission from the winds of very hot stars can be non-thermal in origin. We report here Einstein observations revealing that AS431 has a highly absorbed X-ray spectrum and a relatively strong intrinsic flux of $\geq 5 \times 10^{-12}$ erg cm⁻² s⁻¹, and observations at 20 cm and 6 cm with the National Radio Astronomy Observatory Very Large Array (VLA), showing that it is also a moderately strong radio source (~ 35 mJy). These data, together with optical and infrared observations, suggest a model in which both the radio and X-ray emissions arise in a chaotic stellar wind emerging from a single luminous Wolf-Rayet star.

The Einstein Imaging Proportional Counter⁴ (IPC) pulse-height data (0.5–4 keV), fitted using thermal-plasma emission models (with cosmic abundances)⁵, are shown in Fig. 1. Best fits (χ^2 per degree of freedom ≤ 1) yield a temperature of $kT \geq 0.5$ keV and an absorbing column density of $N_H > 10^{22}$ cm⁻². An intrinsic source flux of $\geq 5 \times 10^{-12}$ erg cm⁻² s⁻¹ is indicated, although, because of the large and uncertain correction for the absorption, values ~ 40 -fold greater than this are allowable by the data. We approximate the flux by this lower limit throughout, despite its being derived from the hardest allowable spectrum ($kT = 5$ keV)—much greater than that observed in any other early-type stellar source. A χ^2 distribution test on the X-ray light curve was consistent with AS431 having constant intensity on time scales ranging from ~ 100 s (limited by counting statistics) to the entire observation length of 5,700 s.

No clear photospheric absorption features (for example, TiO bands) are seen in the 5,500–8,000-Å band of the optical spectrum of the source (Fig. 2), although the interstellar Na-D lines and 6,284-Å band are probably present; this precludes the possibility of AS431 being a late-type emission-line dwarf. The large He I ($\lambda = 5,876, 6,678$ and 7,065-Å)/H α emission-line ratio is, in fact, characteristic of a Wolf-Rayet (W-R) star. Higher-resolution spectra show that the two Na-D absorption features may be partially filled in by the strong He I 5,876-Å emission line, making it difficult to determine the equivalent width of the absorption lines used in estimating the distance to AS431. P. Massey (personal communication) has independently classified this star from his higher resolution spectra as WN8 and it is included in the sixth catalogue of W-R stars (no. 147) by van der Hucht *et al.*⁶.

Multicolour photometry was performed at Kitt Peak National Observatory using the automated filter photometer on the no. 2 0.9-m telescope and the following magnitudes were obtained: $U = 18.37$, $B = 16.72$, $V = 13.84$, $R = 11.00$ and $I = 9.14$. A two-

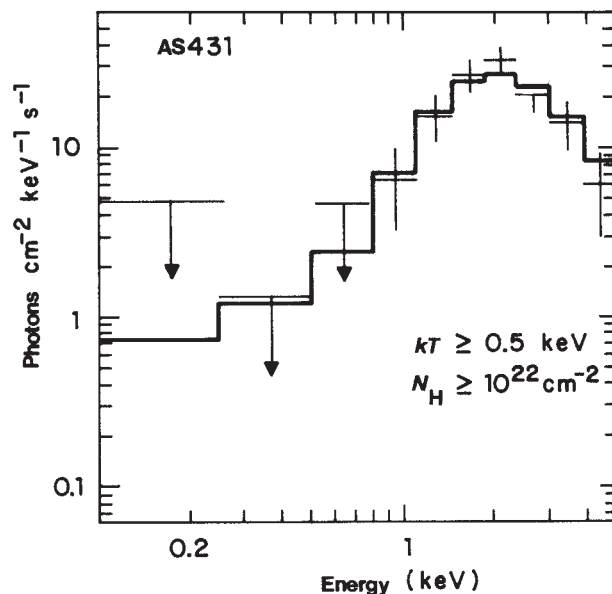


Fig. 1 The IPC pulse-height data for AS431 fitted using thermal plasma emission models. The minimum acceptable values of temperature, kT , and absorbing column density, N_H , are shown.

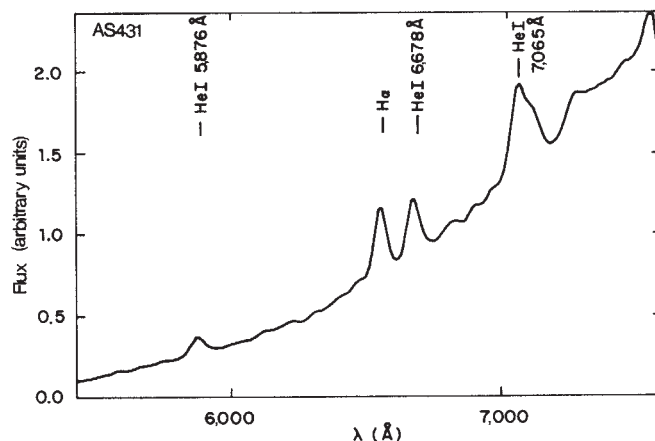


Fig. 2 The optical spectrum of AS431 obtained from the Black Moshannon Observatory 62-inch reflector using a low-dispersion (100–300 Å mm⁻¹) fibre-coupled spectrograph. The resolution is ~ 45 Å and the total integration time is ~ 900 s. The positions of the three He I emission lines and the H α emission line are indicated.

colour ($U-B$ against $B-V$) plot is shown in Fig. 3. As AS431 possesses a W-R-type spectrum, heavy interstellar absorption ($A_V = 7-10$ mag, determined from the standard reddening law⁷) is probably responsible for its place at the extreme lower right of the diagram. This is consistent with the value of A_V determined from comparing the ratio of its total and selective extinctions ($R_V = A_V/E_{B-V}$) with the colour excess (E_{B-V}); the large value of $E_{B-V} \sim 3-4$ (assuming $-0.5 \leq (B-V)_0 \leq -0.2$ for W-R stars⁸), combined with the canonical ratio of $R_V = 3.0 \pm 0.2$ (ref. 9), yields $A_V \sim 8-11$. These values are consistent with the X-ray column density ($N_H > 10^{22}$ cm⁻²) for an absorbing medium with typical interstellar values of dust/gas ratio ($N_H \sim 2 \times 10^{21} A_V$ cm⁻² mag⁻¹)⁹.

Data from the more appropriate narrow band-pass filter system for W-R stars¹⁰⁻¹¹ are also consistent with $A_V \sim 7-11$ and imply a distance of ~ 2 kpc to AS431 (WR 147), which is known to be very close to WRs 144, 145 and 146, members of the heavily reddened Cyg OB2 association ~ 2 kpc¹² distant. At this distance, AS431 would have $-8 \leq M_V \leq -7$, typical of WN8 stars⁶ and its resulting X-ray luminosity (L_X) is $\geq 2.4 \times 10^{33}$ erg s⁻¹,

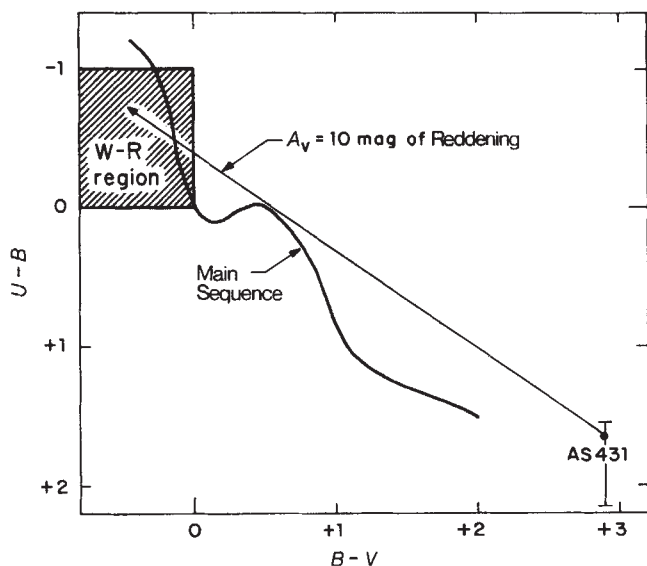


Fig. 3 Two-colour diagram ($U-B$ versus $B-V$). The positions of the main sequence, the W-R region and the standard reddening law as applied to AS431 are indicated. The error bars result from the 'red leak' uncertainty in the U magnitude.

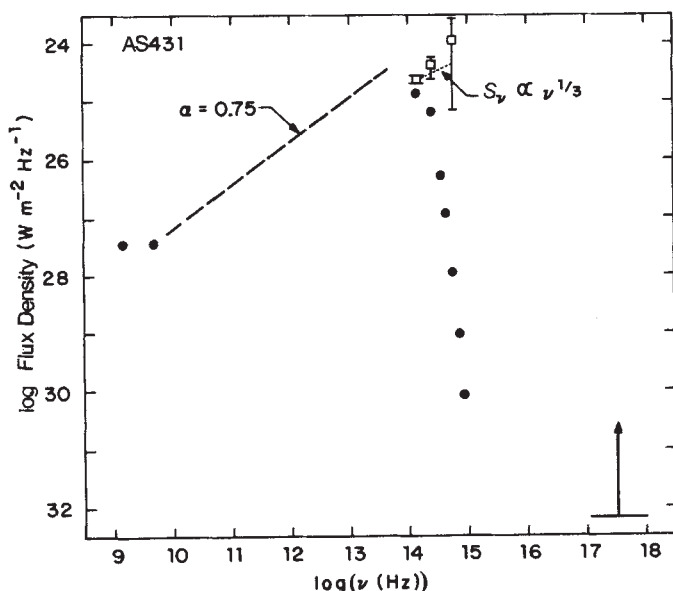


Fig. 4 Plot of the flux density against frequency for all observations of AS431. The filled circles represent the uncorrected fluxes; a few dereddened fluxes (with error bars corresponding to the uncertainty in A_λ) are shown by the open squares. The typical $S_\nu \propto \nu^\alpha$ relation observed for W-R stars between 5 GHz and 10 μm is indicated ($\alpha = 0.75$). The expected $\alpha = 1/3$ optical relation for an accretion source is also shown. The line at the right represents the lower limit to the intrinsic flux in the Einstein X-ray band of 0.5-4.0 keV.

with an L_x/L_{bol} ratio of $\geq 2 \times 10^{-7}$ (assuming a bolometric correction of ~ 4 mag), values which mark AS431 as one of the most X-ray luminous W-R stars yet observed¹³.

Subsequent infrared photometry taken at Kitt Peak with the 2.1-m telescope (10-arc s aperture size) yielded magnitudes of $J = 6.09$, $H = 4.92$ and $K = 4.15$, enabling us to postulate that a dust shell surrounds AS431. A value of $A_V \sim 10$ corresponds to values of $A_J \sim 2.3$, $A_H \sim 1.1$ and $A_K \sim 0.7$ (ref. 14). Having observed $E_{J-K} \geq 1.54$ (assuming $0.2 \leq (J-K)_0 \leq 0.4$ for W-R stars⁸), not all of which can be due to the absorbing material, we suggest that infrared-excess emission (not unusual for W-R stars) accounts for the difference, occurring by either free-free

emission in a gaseous shell or thermal emission from grains in a circumstellar dust shell¹⁵. However, as the dereddened infrared fluxes for AS431 lie above the spectrum usually ascribed to free-free sources ($F_\nu = \text{constant}$)¹⁶, we suggest that a circumstellar dust shell is the source of the infrared excess.

AS431 was also observed at 1.465 GHz (50-MHz bandwidth) in the B-C configuration and at 4.885 GHz (50-MHz bandwidth) in the C-D configuration at the VLA on 11 March and 18 July 1984, respectively. In snapshots lasting ~ 10 min, the integrated fluxes were 36 ± 3 mJy at 20 cm and 38 ± 1 mJy at 6 cm, yielding a spectral index $\alpha \sim 0.05$ and implying, at 2 kpc, a radio luminosity of $L_R \sim 10^{20} \text{ erg s}^{-1} \text{ Hz}^{-1}$. In another survey¹⁷, AS431, when observed (May 1984) at 6 cm with the VLA, giving a flux of ~ 34 mJy, was partially resolved and ~ 20 times brighter than any of the other (~ 20) W-R stars also observed. Using standard thermal-wind-emission models¹⁸ and assuming a terminal-wind velocity $v_\infty \sim 2,000 \text{ km s}^{-1}$, $\text{He}^{2+}/\text{He}^+ \sim 1$ and $\text{He}/\text{H} \gg 1$ (ref. 19), one obtains a mass-loss rate for AS431 of $\dot{M} \sim 3.9 \times 10^{-4} M_\odot \text{ yr}^{-1}$, about an order of magnitude larger than the average value for other W-R stars¹⁹. The nature of the radio emission is uncertain, as, being extended, it suggests a thermal-wind model, whereas the large $\dot{M}$ and the flat spectrum otherwise imply a non-thermal source.

One possible mechanism is accretion of the high-velocity stellar wind onto a compact companion²⁰. W-R stars in binary systems with compact companions (WN+c systems) are not uncommon²¹. If AS431 were such a system, the large amounts of both X-ray and radio emission could be explained by the combination of thermal emission from the wind and non-thermal emission from accretion and would no longer be anomalous. Figure 4 shows the spectrum of AS431 from the radio to X-ray regions. Accretion models²² suggest $\alpha = 2$ in the radio regime and $\alpha = 0.33$ (dotted line) for the optical and near-infrared radiation, but neither of these agree with our observations, where $\alpha_{\text{radio}} \sim 0$ and $\alpha_{\text{optical}} \sim 1$. Also, variability has not been observed in either regime, although it is typical of most accretion sources.

An alternative non-thermal mechanism is the acceleration of relativistic electrons by shocks near the base of the wind²³. In such chaotic stellar wind models, reasonable stellar parameters yield a flat spectrum above radio frequencies of ~ 1 GHz and, in this case, the infrared spectrum will be dominated by thermal emission from the wind. The dashed line ($\alpha \sim 0.75$) in Fig. 4 represents the typical $S_\nu \propto \nu^\alpha$ relation (where S_ν is the flux at frequency ν and α is the spectral index), between 5 GHz and 10 μm , for W-R stars¹⁹, which is in reasonable agreement with the data for AS431. In addition, the predicted radio flux (assuming $\dot{M}$ for W-R stars is $\sim 2 \times 10^{-5} M_\odot \text{ yr}^{-1}$) is within 50% of that observed; the model further predicts that some flat-spectrum synchrotron sources ought to be resolvable. As these embedded-shock models²⁴ have been shown to describe adequately the observed X-ray emission from hot stars as well²⁵, we are compelled to consider them for the rather high X-ray and radio emission of AS431, whereas for weaker W-R stars, simple thermal models apparently suffice.

The Infrared Astronomical Satellite all-sky survey should reveal AS431 as a strong source (5-25 Jy) at all wavelengths (12, 25, 60 and 100 μm) and yield more accurate determinations of the contribution of the dust shell to the extinction and infrared emission. Higher-resolution radio observations could potentially distinguish between thermal and non-thermal models by determining the surface brightness profile of the source.

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1. Caillault, J.-P., Chanan, G. A., Helfand, D. J., Margon, B. & Downes, R. *Bull. Am. astr. Soc.* **14**, 951 (1982).
2. Takalo, L., Nousek, J., Caillault, J.-P. & Helfand, D. J. *Bull. Am. astr. Soc.* **16**, 469 (1984).
3. Merrill, P. W. & Burwell, C. G. *Astrophys. J.* **112**, 72-89 (1950).
4. Giacconi, R. *et al.* *Astrophys. J.* **230**, 540-550 (1979).
5. Raymond, J. C. & Smith, B. W. *Astrophys. J. Suppl.* **35**, 419-439 (1977).
6. van der Hucht, K. A., Conti, P. S., Lundstrom, I. & Stenholm, B. *Space Sci. Rev.* **28**, 227-306 (1981).
7. Hiltner, W. A. & Johnson, H. L. *Astrophys. J.* **124**, 367-378 (1956).
8. Johnson, H. L. *A. Rev. Astr. Astrophys.* **4**, 193-206 (1966).
9. Spitzer, L. Jr *Physical Processes in the Interstellar Medium*, 149-171 (Wiley, New York, 1978).
10. Massey, P. *Astrophys. J.* **281**, 789-799 (1984).
11. Smith, L. F. *Mon. Not. R. astr. Soc.* **140**, 409-433 (1968).
12. Abbott, D. C., Biegging, J. H. & Churchwell, E. *Astrophys. J.* **250**, 645-659 (1981).
13. Sanders, W. T., Cassinelli, J. P., Myers, R. V. & van der Hucht, K. *Astrophys. J.* (submitted).
14. Persson, S. E., Aaronson, M., Cohen, J. G., Frogel, J. A. & Matthews, K. *Astrophys. J.* **266**, 105-129 (1983).
15. Cohen, M., Barlow, M. J. & Kuhl, L. V. *Astr. Astrophys.* **40**, 291-302 (1975).
16. Gallagher, J. S. & Ney, E. P. *Astrophys. J. Lett.* **204**, L35-L39 (1976).
17. Abbott, D. C. & Biegging, J. H. *Bull. Am. astr. Soc.* **16**, 508 (1984).
18. Wright, A. E. & Barlow, M. J. *Mon. Not. R. astr. Soc.* **170**, 41-51 (1975).
19. Barlow, M. J., Smith, L. J. & Willis, A. J. *Mon. Not. R. astr. Soc.* **196**, 101-110 (1981).
20. Abbott, D. C., Biegging, J. H. & Churchwell, E. *Astrophys. J.* **280**, 671-678 (1984).
21. Moffat, A. F. J. *IAU Symp.* **99**, 263-275 (1982).
22. Pringle, J. E. A. *Rev. Astr. Astrophys.* **19**, 137-162 (1981).
23. White, R. L. *Astrophys. J.* (submitted).
24. Lucy, L. B. & White, R. L. *Astrophys. J.* **241**, 300-305 (1980).
25. Cassinelli, J. P. & Swank, J. *Astrophys. J.* **271**, 681-690 (1983).

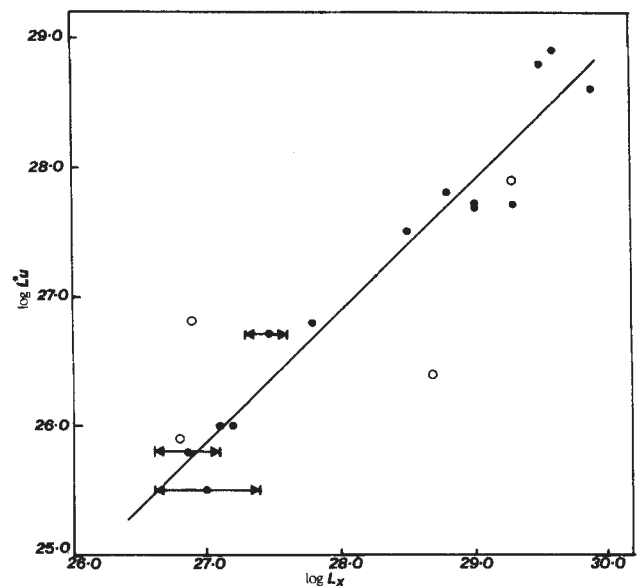


Fig. 1 A plot of the time-averaged flare energy in the U band, L_u^* , the 'quiescent' X-ray flux, L_x . Both L_u^* and L_x are in units of erg s^{-1} . Open circles represent data points based on <15 h of observation and thus are probably uncertain.

Ultraviolet radiation from stellar flares and the coronal X-ray emission for dwarf-Me stars

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The origin of chromospheric and coronal emission is thought to derive ultimately from magnetic fields generated by a dynamo mechanism involving the interaction of rotation and convection¹. Although these magnetic fields are difficult to detect directly, the dramatic stellar flares, believed to be the result of magnetic reconnection, have been observed for many years on dwarf Me (dMe) stars. Here we correlate Einstein observations of the X-ray flux of quiescent dMe stars with the time-averaged energy emitted by flares in the Johnson-U band, showing that the X-ray energy emitted by the coronae of these stars is about an order of magnitude greater than the U-band flare energy. From our estimate of the ratio of the total radiation emitted to the U-band flux, it is possible that, if a similar amount of energy were dissipated in the stellar atmosphere, then the observed flare events could heat the coronae of these stars.

In a survey of X-ray fluxes from the Einstein satellite², those for the dMe stars varied over three orders of magnitude. To investigate the relationship between this flux and flare activity, we compiled a list of stars (Table 1) for which both the flare-activity rate in the U band and the 'quiescent' X-ray flux were known. The time-averaged flare energy, L_u^* , is defined as the summation of the total flare energy divided by the total monitoring time and L_x is the X-ray flux observed by the Imaging Proportional Counter on board Einstein, in the energy range 0.2-4 keV, while the star was judged to be quiescent. No attempt was made to correct the U data for detection threshold, which, despite the use of telescopes of different size, should not affect seriously the data, given sufficient monitoring hours to determine the rate. Furthermore, as large flares dominate the time-averaged energy, differences in the threshold are not a serious concern. Spectral classification is from refs 3-6 and, except Gl 15A and Gl 825, all stars exhibit their hydrogen-Balmer lines in emission. A straight line through the data plotted in Fig. 1 (excluding uncertain points for Gl 234A and Gl 735) has the form $\log L_x = 1.0 \log L_u^* + 1.12$.

Of the two recorded observations of flaring on Gl 735^{7,8}, doubt exists for one of these⁷ concerning the determination of the time-averaged flare energy; the other⁸ was of 5 Johnson-B-band flares during 30 h of monitoring. Using the relationship $L_u = 1.2 L_B$ (ref. 9), these observations would imply $\log L_u^* = 27.59$,

which is in excellent agreement with the mean relation for the other stars in Fig. 1. The existing data for Gl 234A are based on only 8.8 h of monitoring¹⁰. One of us (J.G.D.) has recently observed Gl 234A with the 0.75 m telescope at the South African Astronomical Observatory, but because of poor weather the accumulated monitoring time was only 4 h, during which the time-averaged flare energy was an order of magnitude less than that given in Table 1. Thus, further observations for these two stars are required.

Before discussing the implications of the relationship between L_x and L_u^* , we should address the uniqueness of relationships involving X-ray flux and certain activity parameters such as rotation rate and Ca(II) flux. Because of the sensitivity threshold of present X-ray detectors, the data for nearby dwarfs are highly selective in that the intrinsically-feint sources are only seen in close proximity to the Solar System and the less common intrinsically-brighter sources are only seen further away¹¹. Thus we do not have a genuine volume-limited sample if detection in X rays is the criterion of selection; but, for the data plotted in Fig. 1, the data are selected not according to their X-ray flux but by optical observers monitoring for flares in the U band. In compiling Table 1 we have listed all the data for U band monitoring of known flares and for all of these stars the X-ray flux has been measured by Einstein; our sample-selection criterion is therefore the detectability of optical flares, rather than detection of X-ray emission. Exactly how the optical observers have selected their objects is difficult to assess, but, as most use relatively small telescopes, a magnitude limit of $B \sim 12$ is probably appropriate. The dMe stars in our sample range from $V = 8-13.5$, that is, a difference of over two orders of magnitude in optical brightness, randomly distributed in distance. Hence we believe the correlation in Fig. 1 is significant and not an artefact of selection effects.

The slope of the line in Fig. 1 is unity, which implies that $L_x \propto L_u^*$, that is the quiescent coronal emission is proportional to the time-averaged energy of the flares observed in the U band. From the solar analogy, any aspect of stellar activity, such as L_u^* , should be correlated with the coronal X-ray emission, but this correlation was not expected to be linear over a range of over three orders of magnitude—an empirical result for which the physical mechanism is not yet understood. Two possibilities are: (1) that both the flare rate and the X-ray emission are the result of 'magnetic activity', but that L_x is not a direct result of L_u^* and (2) that the X-ray emission is a direct result of heating by flares and therefore $L_x \propto L_u^*$. In the first case the mechanism responsible for the optically-detected flares may parallel a

Table 1 Flare stars

Star	Name	Spectral type	$\log L_x$	$\log L_u^*$	T_u
Gl 182	V1005 Ori	dM0.5e	29.5 ²¹	28.8 ²¹	62.1
Gl 388	AD Leo	dM3.5e	29.0 ¹	27.7 ²²	48.0
Gl 285	YZ CMi	dM4.5e	28.5 ²³	27.5 ^{24,25}	51.6
Gl 65	UV Cet	dM6e	27.3–27.6 ²	26.7 ^{24,26}	32.9
Gl 278c	YY Gem	dM1e	29.6 ²	28.9 ²⁴	74.9
Gl 896	EQ Peg	dM4e	28.8 ²	27.8 ²⁴	51.5
Gl 406	CN Leo	dM6.5e	26.6–27.1 ^{2,13}	25.8 ²⁴	29.0
Gl 867A	FK Aql	dM1e	29.0 ¹	27.7 ²⁷	21.3
Gl 825		dM1	27.2 ¹	26.0 ²⁸	26.0
Gl 735	V1285 Aql	dM3e	28.7 ²⁹	26.4 ⁷	14.5
Gl 803	AU Mic	dM2.5e	29.9 ³⁰	28.6 ^{7,31}	32.1
Gl 15A		dM2.5	27.1 ²	26.0 ⁶	48.3
Gl 551	Prox Cen	dM5e	26.6–27.4 ^{2,13}	25.5 ³²	39.3
Gl 229		dM2.5	26.8 ²⁹	25.9	6.7
Gl 166c	40 Eri C	dM4e	27.8 ²	26.8	16.3
Gl 234A	Ross 614A	dM4.5e	26.9 ¹	26.8 ¹⁰	8.8
Gl 799A	AT Mic	dM4.5e	29.3 ¹	27.9 ¹⁰	11.3
Gl 644	Wolf 630	dM3.5e	29.3 ²	27.7 ¹⁰	46.5

A tabulation of flare stars, whose 'quiescent' X-ray flux L_x and time-averaged flare energy L_u^* in the Johnson-U band is known. The total number of hours T_u , spent monitoring in the U band is also given. Both L_x and L_u^* are in erg s^{-1} . Superscripts refer to the reference.

related, but not identical, mechanism producing the coronal heating. For example, the coronal heating may arise from the reconnection of small magnetic-loop structures and the dramatic flares seen in the U band from the reconnection of much larger and more energetic loops.

Two aspects of our data may influence our understanding of the mechanism responsible for coronal heating: first, the quantitative comparison of the energy of optical flares with that radiated by the corona in X rays; second, the scatter of the data points in Fig. 1. Rewriting the logarithmic relation as $L_x = 13.2 L_u^*$ we see that the quiescent X-ray flux from the coronae of the dMe stars is only about an order of magnitude greater than the time-averaged flare output in the U band, which raises the question, implied by (2) above, of whether all the energy contained in the corona and radiated in X rays could originate in stellar flares. Although to answer this, we must be able to assess the ratio of the total energy released in a stellar flare to that emitted in the U band, this ratio cannot be determined directly for any flares yet observed on dMe stars. Even though stellar flares have now been observed in X rays (Einstein satellite), in the ultraviolet (IUE), in radiowaves (VLA) and optically, in no case have observations been made over the entire range. Several flares, however, have now been observed optically, simultaneously, by IUE and the VLA radio telescope¹² and by IUE and the Einstein satellite¹³, but as these observations were of different flares and on different stars, it is doubtful whether a real value for L_{total}/L_u could be derived from them, particularly in view of the complexity and variety of stellar flares. Nevertheless, even an order of magnitude result would be of some interest.

Using the result⁹ $L_{\text{opt}} = 2.4 L_u$, the relative energies in different wavebands for flares on YZ CMi, AD Leo and Prox Cen^{12,13} and data for a solar flare¹⁴, we estimate that the total optical, ultraviolet and X-ray energy emitted from a flare is $\sim 7 L_u$. But, in converting to time-averaged energy, we must multiply by a factor of 2 to include the unseen flares which occur on the opposite hemisphere to that of the observer, that is, $L_{\text{total}}^* = 14 L_u^*$. Thus, $L_x \approx L_{\text{total}}^*$ from which it follows that, if an approximately equal amount of energy to that emitted by flares in electromagnetic radiation were to be deposited in the coronae of the dMe stars, for instance, by shock waves, there would be sufficient energy in the flares alone to heat the coronae of these stars. In fact the amount of mechanical energy deposited in the stellar atmosphere by the flare event may far exceed the radiated energy and the only flare for which the former has been estimated, the solar flare observed by Skylab on 5 September 1973, has indeed a ratio of mechanical to radiated energy^{14,15} of 50:1.

The energy requirements of the solar corona are perhaps provided by high-velocity jets of material from the chromos-

phere¹⁶. These jets, observed with high-spatial and temporal resolution have typical velocities of 400 km s^{-1} , are confined within a small area, $\leq 2''$ diameter and are thought to be exploding small loops, perhaps involving the reconnection of two loops, as proposed for flare theory¹⁷. As their velocities are much greater than the Alfvén velocity, shock waves will be generated in the corona, resulting in coronal heating. Evidence for high-velocity ($V \sim 1,000 \text{ km s}^{-1}$) mass motions during flares on dMe stars has been observed recently¹².

Returning to the scatter of the data in Fig. 1, we see that the individual points do not deviate by more than a factor of two from the straight line. As L_x and L_u^* were measured at different epochs, the small scatter of the data points from the mean relation implies that any short-term (days) or long-term variability (years) in the X-ray flux be less than a factor of two. This is consistent with a maximum degree of variability of less than a factor of 3 for some of these stars¹⁸ and is much less than the factor of ~ 6 for the rotational modulation in the solar quiescent-X-ray emission¹⁹, suggesting that the coronae of dMe stars are more uniform than their solar counterpart which, from X-ray imaging and rotational modulation of X rays, is seen to be highly inhomogeneous.

This low variability in the quiescent X-ray emission implies either than the heating rate for the stellar coronae is relatively constant, or the heat capacity of the coronae is sufficient to smooth the release of energy that accompanies even the largest flares. The irregularity of stellar flares suggests that they are not the sole mechanism for heating the corona. Thus, L_u^* and L_x may be well correlated because flares are only an extreme example of the processes keeping coronae hot, that is, flares result from magnetic reconnection on a large scale whereas coronal heating results from reconnection on a small scale²⁰. Nevertheless, the tight linear correlation of L_u^* and L_x and the remarkable quantitative similarity between L_{total}^* and L_x suggest that flares play a direct role in the heating of the corona.

Note added in proof: It has come to our attention that a quite similar but independent paper on this topic by D. R. Whitehouse is to appear in *Astronomy and Astrophysics*.

Received 6 July; accepted 23 November 1984.

1. Golub, L. *IAU Colloq.* **71**, 83–108 (1983).
2. Vaiana, G. S. et al. *Astrophys. J.* **245**, 163–182 (1981).
3. Joy, A. H. & Abt, H. A. *Astrophys. J. (Suppl.)* **28**, 1–18 (1974).
4. Woolley, R., Epps, E. A., Penston, M. J. & Pocock, S. B. *R. Obs. Ann.* **5**, (1970).
5. Byrne, P. B., Doyle, J. G. & Menzies, J. *Mon. Not. R. astr. Soc. (in the press)*.
6. Pettersen, B. J. & Griffin, R. F. *Observatory* **100**, 198–202 (1980).
7. Byrne, P. B., Doyle, J. G., Butler, C. J. & Andrews, A. D. *Mon. Not. R. astr. Soc. (in the press)*.
8. Shakhovskaya, N. I. *Inf. Bull. Var. Stars* No. 487 (1970).
9. Lacy, C. H., Moffet, T. J. & Evans, D. S. *Astrophys. J. (Suppl.)* **30**, 85–86 (1974).
10. Kunkel, S. W. *Astrophys. J. (Suppl.)* **25**, 1–36 (1973).

11. Rosner, R. *IAU Symp.* **102**, 279-300 (1983).
12. Rodono, M., et al. *Proc. 4th Eur. IUE Conf.* (in the press).
13. Haisch, B. M. et al. *Astrophys. J. Lett.* **242**, L99-L103 (1980).
14. Cranfield, R. C. et al. in *Solar Flares* (ed. P. A. Sturrock) 451-469 (Colorado University Press, 1980).
15. Webb, D. F. et al. in *Solar Flares* (ed. P. A. Sturrock) 471-499 (Colorado University Press, 1980).
16. Brueckner, G. E. & Bartoe, J. D. F. *Astrophys. J.* **272**, 329-348 (1983).
17. Priest, E. R. in *Solar Active Regions* (ed. F. Q. Orall) (Colorado University Press, 1981).
18. Vaiana, G. S. *IAU Symp.* **102**, 165-186 (1983).
19. Vaiana, G. S. & Rosner, R. A. *Rev. Astr. Astrophys.* **16**, 393-428 (1978).
20. Mullan, D. J. *Irish Astr. J.* **15**, 147-151 (1981).
21. Byrne, P. B., Doyle, J. G. & Butler, C. J. *Mon. Not. R. astr. Soc.* **206**, 907-918 (1984).
22. Pettersen, B. J., Coleman, L. A. & Evans, D. S. *Astrophys. J. (Suppl.)* **54**, 375-386 (1984).
23. Kahler, S. W. et al. *Astrophys. J.* **252**, 239-249 (1982).
24. Moffett, T. J. *Astrophys. J. (Suppl.)* **29**, 1-42 (1974).
25. Ichimura, K. & Shimizu, Y. *Inf. Bull. Var. Stars* No. 1420 (1978).
26. Panov, K. P., Asteriadis, G. & Mavridis, L. N. *Inf. Bull. Var. Stars* No. 2359 (1983).
27. Byrne, P. B. & McFarland, J. *Mon. Not. R. astr. Soc.* **193**, 525-532 (1980).
28. Byrne, P. B. *Mon. Not. R. astr. Soc.* **195**, 143-147 (1981).
29. Agrawal, P. C., Rao, A. R. & Sreekantan, B. V. *IAU Colloq.* **71**, 125-129 (1983).
30. Caillault, J. P. *Astr. J.* **87**, 558-562 (1982).
31. Busko, I. C. & Torres, C. A. O. *Inf. Bull. Var. Stars* No. 1186 (1976).
32. Walker, A. R. *Mon. Not. R. astr. Soc.* **195**, 1029-1035 (1981).

Solar burst with millimetre-wave emission at high frequency only

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Solar burst emission data at shorter millimetre wavelengths have been based on a few events measured with low sensitivity and relatively poor time resolution¹⁻³. We present here the first high sensitivity and high time-resolution observations taken simultaneously at 90 GHz ($\lambda = 3.3$ mm) and at 30 GHz ($\lambda = 10$ mm). These have identified a unique impulsive burst on 21 May 1984 with fast pulsed emission that was considerably more intense at 90 GHz than at lower frequencies. Hard X-ray time structures at energies above 25 keV were almost identical to the 90 GHz structures to better than 1 s. The structure of the onset of the major 90-GHz burst coincided with the hard X-ray structure to within 128 ms. All 90-GHz major time structures consisted of trains of multiple sub-second pulses with rise times as short as 0.03 s and amplitudes that were large compared with the mean flux. When detectable, the 30-GHz sub-second pulses had smaller relative amplitude and were in phase with the corresponding 90-GHz pulses.

Past observations at centimetre-millimetre wavelengths indicate that certain bursts can display turnover frequencies which are higher than 30 GHz ($\lambda < 10$ mm)⁴⁻⁹. One very large burst exhibited a spectral 'flattening' in the range 30-71 GHz (refs 4, 5), which was also found for a few other events⁷. Another peculiar event showed emission at 90 GHz ($\lambda = 3.3$ mm) but no emission has been revealed by investigations in the microwave region⁹. More recently, 80 GHz ($\lambda = 3.75$ mm) and 92.5 GHz ($\lambda = 3.2$ mm) solar measurements were started in Japan¹⁰ and Switzerland (A. Magun, personal communication), respectively, with 10 SFU (solar flux unit) sensitivity (1 SFU = 10^{-22} W m⁻² Hz⁻¹) and 0.3-s time resolution.

Observations were made with the Itapetinga 13.7-m radome-enclosed antenna, used for the first time at 90 GHz ($\lambda = 3.3$ mm) for solar measurements in May 1984. The antenna aperture efficiency at that frequency is about 25% at the zenith and decreases with elevation angle¹¹. The antenna beam size was also dependent on elevation angle, being ~80 arc s from zenith to 50° of elevation, and enlarging rapidly for lower elevation angles¹¹. For solar elevation angles of 60°-10°, the corresponding sensitivity in the antenna main beam ranged from 0.07 to 0.25 SFU with a time constant of 1 ms. This represents an improvement of more than two orders of magnitude in both sensitivity and time resolution compared with previous observations at this frequency.

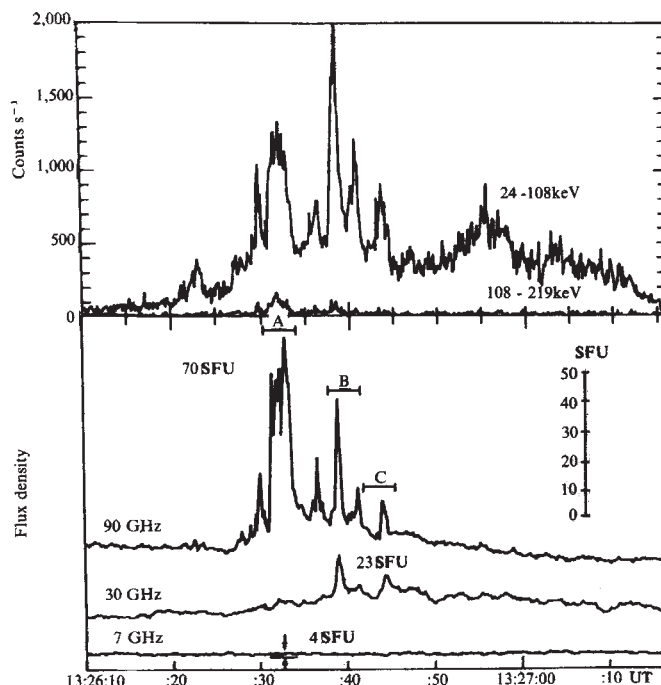


Fig. 1 The solar burst on 21 May 1984 at 13 h 26 min UT, as observed by HXRBS in X-rays at 24-108 keV and 108-219 keV, and in millimetre/centimetre wavelengths at 90, 30 and 7 GHz. The SFU scale is subject to an uncertainty of a factor of <2. Major structures in the time profiles labelled A, B, and C are plotted on expanded time scales in Figs 2 and 3.

Simultaneous observations were made at 30 GHz using the same antenna with a sensitivity of 0.03 SFU with a time constant of 1 ms and beam size of ~3 arc min, independent from elevation angle. Active regions were tracked at a spatial position referred to their maximum intensity positions defined by the antenna beams, and the flux scales were established by assuming that the burst sources were coincident in space with the active region maximum intensity position. Previous experience at other millimetre-microwave frequencies (22 GHz and 44 GHz) indicated that bursts usually occur at about the same position of the active region maximum intensity position. The separation of the 90-GHz and 30-GHz beams in space was 3 arc min, and the tracking accuracy was better than 2 arc s r.m.s. The positions of the two antenna beams with respect to the active region maximum intensity position were well known. As the beam shapes were also well known, it was possible to establish the flux scale factors at both frequencies. The main uncertainty factor was the lack of knowledge of the burst source location with respect to the active region maximum intensity location. This uncertainty, however, should not exceed 50% in the most extreme case. Observations were also made at 7 GHz by a patrol radio telescope using a 1.5-m antenna with a sensitivity of 3 SFU and a time constant of 20 ms. Other details of the observational setup at Itapetinga are given elsewhere¹².

Simultaneous observations were also made using the hard X-ray burst spectrometer (HXRBS) on the Solar Maximum Mission (SMM) satellite¹³. A 15-channel counting rate spectrum covering the energy range 24-400 keV was obtained with this instrument every 128 ms throughout the flare.

A 90-GHz impulsive burst occurred on 21 May 1984 at 13.26 UT with the Sun at 40° of elevation. At that elevation angle the antenna beam sizes at 90 GHz and 30 GHz were 1.7 arc min and 3 arc min, respectively. The positions of the beams with respect to the active region maximum intensity position were displaced by 1.5 arc min at both 90 and 30 GHz. The burst demonstrated the unique characteristic of emission at the shortest millimetre-wavelengths only, so it cannot be included in any known class of solar radio burst. Figure 1 shows the impulsive

event at 90 GHz, 30 GHz and 7 GHz and in two hard X-ray energy ranges, 24–108 keV and 108–219 keV. The millimetre-wave emission spectrum suggests a turnover frequency as high as or higher than 90 GHz. The negligible rise at 7 GHz of 4 SFU was confirmed by data obtained at 2.8 GHz showing a <3 SFU increase at the same time¹⁴, and by data plots obtained from Sagamore Hill Radio Observatory (E. W. Cliver, personal communication) at various frequencies, ranging from 0.6 to 15.4 GHz, exhibiting fluxes ≈ 4 SFU, and a rise at 0.4 GHz (~ 30 SFU).

The first major impulsive component was observed at 90 GHz almost 6 s before the first impulsive structure at 30 GHz. Assuming that the flux scales are correct, the first structure, A, in Fig. 1, reached ~ 70 SFU at 90 GHz and 6 SFU at 30 GHz. The second major structure, B, in Fig. 1 reached 56 SFU at 90 GHz and 23 SFU at 30 GHz, and the third structure, C, reached 24 SFU at 90 GHz and 14 SFU at 30 GHz. The power law spectral index α in the relation $S \propto f^\alpha$, where S is the flux density and f is the frequency, is estimated from the 30 and 90 GHz fluxes to be 2.2 for A, 0.8 for B, and 0.5 for C.

The time variation of the flux ratio between 90 and 30 GHz, for different major burst structures, cannot be explained by changes in burst source position with respect to the antenna beams. The negligible intensity at 30 GHz for structure A (~ 6 SFU) was also confirmed by full-Sun data at 15.4 GHz (≈ 4 SFU) (E. W. Cliver, personal communication). Furthermore, the very good proportionality between all the time structures of the 90 GHz emissions and X-ray intensities could not be conceived with different structures occurring at spatial positions with significant displacements. Therefore, the observed variations in spectral ratio seem to be genuine.

The hard X-ray time profiles in Fig. 1 show a striking similarity to the 90-GHz time profile. Features coincide to within the 128-ms time resolution of the hard X-ray observations. It is interesting to compare the relative amplitudes of structures A and B at different X-ray energies and millimetre-wave frequencies. For the 24–108 keV X rays and the 30-GHz emission, B is more intense than A, whereas for the 108–219 keV X rays and the 90-GHz emission, the converse is true. This suggests that the 90-GHz emission comes primarily from the higher energy electrons or at least from electrons with a harder spectrum. The power law spectral index γ in the relation $I \propto \epsilon^{-\gamma}$, where I is the X-ray flux and ϵ is the photon energy, was estimated to be 3.2 for A, 3.8 for B, and 4.5 for C. Almost 1 min after C, a sudden increase in γ to 5.8, corresponding to a secondary rise in the hard X-ray flux, was accompanied by weak fluctuations observed at 7 and 2.8 GHz (ref. 14). This was apparently the beginning of a conventional microwave burst with a peak frequency at 8–15 GHz and a peak flux of 40 SFU at 8.8 GHz occurring at 13.31 UT (E. W. Cliver, personal communication), some 4 min after the initial hard X-ray and 90 GHz spikes. A minor peak in the >24 keV X-ray flux also occurred at this time but with a HXRBS counting rate of only 300 counts s^{-1} compared with 1,630 counts s^{-1} at the time of the earlier, more impulsive spikes.

The high time resolution achieved in the 90 and 30 GHz observations allowed the detection of even faster intensity variations than are evident in Fig. 1. All the major impulsive structures appeared as trains of several very fast peaks when plotted on an expanded time scale. Figure 2 shows an expanded plot of A at 90 GHz and in 24–219 keV X rays. Rapid variations are detected in the 90-GHz intensity with at least 13 resolved peaks in just over 2 s. The e -folding rise time of the initial peak is ~ 0.03 s. The detection of similar rapid variations in hard X rays was precluded in this case by the 128 ms time resolution of the observations. However, other events have been detected by HXRBS with a time resolution of 10 ms showing equally rapid fluctuations in the hard X-ray flux¹⁵. In the present case, Fig. 2 shows that the initial rise of A in hard X rays is simultaneous with the rise of the 90-GHz profile to within the 128-ms resolution of the X-ray observations. The overall X-ray and 90-GHz time profiles, however, are different. This can be attributed to the

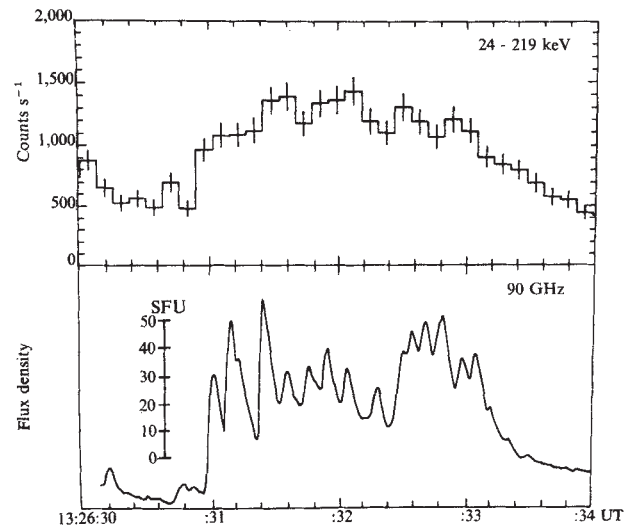


Fig. 2 The 4-s expansion of the 24–440 keV X-ray and 90 GHz intensities in structure A of the burst shown in Fig. 1. The vertical bars on the X-ray plot indicate $\pm 1\sigma$ uncertainties in the measured rate based on the square-root of the observed number of counts in each interval. The horizontal bars indicate the 128-ms time intervals over which the counting rates, corrected for instrumental dead time, were determined. The time constant used for the 90-GHz plot was 10 ms and the system noise was comparable to the thickness of the line.

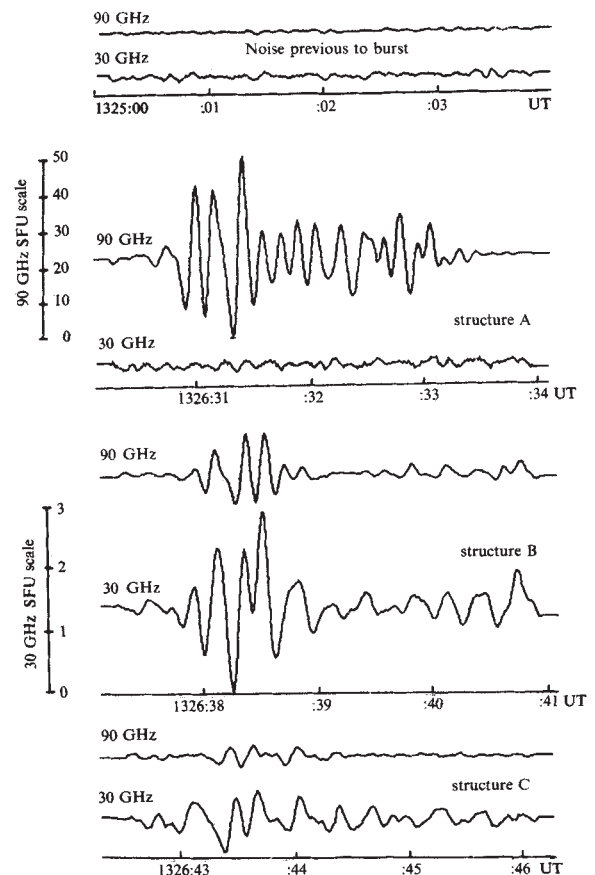


Fig. 3 Major burst structures at 90 and 30 GHz, labelled A, B and C in Fig. 1, plotted on an expanded time scale with a running mean subtracted from the measured fluxes. The flux variations in a 4-s interval are plotted for each structure at the two frequencies with a similar plot at the top of the figure showing the system noise in a 4-s interval, 1 min before the start of the burst. The vertical scale for the 90-GHz plot is the same for all four intervals and is given to the left of the curves for A. Similarly, the common scale for the 30-GHz plots is given to the left of the curves for B.

limited time resolution of X rays, which smoothes out all repetitive time structures that are faster than ~ 128 ms. Hard X-ray plots, similar to Fig. 2, but taken at higher energy, would produce the same smoothing effect with large error bars owing to the smaller counting rates.

To show these rapid millimetre-wave fluctuations more clearly, the gradually varying component of the 90- and 30-GHz flux has been removed in Fig. 3 by subtracting a running mean. Plots for the three major structures A, B and C are shown together with a similar plot for a 4-s interval before the burst. Note that the system noise amplitude is negligible compared with the pulse sizes.

Figure 3 shows that the rapid fluctuations in A are evident only at 90 GHz. The pulse repetition rate is ~ 5 Hz but a Fourier analysis¹⁶ shows that there is significant power at frequencies extending from 1 to 8 Hz. The relative amplitudes of the pulses range from 30 to 50% of the mean flux. Such pulses are barely noticeable at 30 GHz, however, and they have much smaller amplitude. The situation for B is different with pulse trains evident at both 90 and 30 GHz. The five pulses observed in ~ 1 s starting at 13 h 26 min 38 s UT are essentially in phase at the two frequencies but the relative amplitudes are still much larger at 90 GHz (60%) than at 30 GHz (1%). Structure C in Fig. 3 also shows a train pulses but with lower relative amplitude at both frequencies.

The pulse trains at 30 GHz with relative amplitudes of $\leq 1\%$ are comparable with the ultrafast structures observed for other bursts at 22 and 44 GHz (refs 17, 18). Similar sub-second pulses have been detected in hard X rays coincident with microwave pulses at these same frequencies¹⁹ but the relative amplitudes of the hard X-ray pulses were 30–40%, comparable with the amplitudes of the 90 GHz pulses reported here. A comparison of sub-second time structures in solar bursts, obtained with about 100-ms time resolution simultaneously at 10.6 GHz and hard X rays²⁰, have shown good correspondence, with possible delays for microwaves relative to hard X-rays of ~ 200 ms.

The apparent brightness temperature of the burst source at millimetre-waves can be estimated if some assumptions are made about the velocity scales involved at the pulses acceleration site. If we assume that the speed of light is a limit, the scale for the 30-ms rise times becomes $L < 9 \times 10^8$ cm (or apparent size $\theta_s < 13$ arc s). If we assume Alfvén speeds (10^8 cm s⁻¹), we obtain $L < 3 \times 10^6$ cm (or $\theta_s < 0.04$ arc s). The apparent brightness temperature T_B is related to the flux density S by the relationship $S = (2kT_B/\lambda^2)(L/D)^2$, where k is the Boltzmann constant, λ is the wavelength, and $D = 1 \text{ AU} = 1.5 \times 10^{13}$ cm. For structure A pulses, the fluxes are 70 SFU and 6 SFU, at 90 and 30 GHz, respectively. We obtain comparable brightness temperatures, at both frequencies, of $\sim 10^5$ K (speed of light) and 10^{10} K (Alfvén speed), respectively.

The class of solar bursts emitting at the shortest millimetre wavelengths imposes important constraints on existing models of the mechanisms of particle acceleration in the impulsive phase. Measurements at lower frequencies only, such as 30 GHz alone, would give the wrong idea that microwaves are not well correlated, or delayed, relative to hard X rays. A thermal bremsstrahlung burst component suggested for the higher frequencies⁷ requires burst sizes ($> 10^9$ – 10^{10} cm) which are too large for the time scales found here, even assuming the extreme limit of the speed of light expansion. The assumption of simple synchrotron emission, with a turnover frequency at ~ 90 GHz, would imply unrealistically high magnetic fields^{4, 21}. If we adopt formulae for mildly relativistic electrons²², interpretations may be found, but an extreme value must be assumed for one of the three basic parameters: magnetic field, electron density or scale size (for example, densities $\sim 10^{11}$ cm⁻³, maximum size scale $\sim 10^9$ cm, requiring magnetic fields $> 10^3$ G). In the thermal model the temperature estimated from the hard X-ray spectrum (5×10^8 K) is much higher than the radio-brightness temperature estimated for the larger scale-size assumption (speed of light). To reconcile the temperatures, the source size should be smaller and densities higher, for plausible magnetic fields. For assump-

tion of Alfvén speed for expansion, however, the scale sizes appear to be too small, and the brightness temperatures too high to be interpreted with the above models. Further studies are required to establish a realistic compromise between the physical parameters involved. A study on possible interpretations of the burst spectral component at millimetre wavelengths is in preparation.

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- Coates, R. J. *Proc. natn Electron. Conf.* **13**, 1–12 (1957).
- Coates, R. J. *Proc. IRE* **46**, 122–126 (1958).
- Croom, D. L. & Powell, R. J. *Nature* **221**, 945–947 (1969).
- Croom, D. L. *Solar Phys.* **15**, 414–423 (1970).
- Cogdell, J. R. *Solar Phys.* **22**, 147–149 (1972).
- Feix, G. J. *geophys. Res.* **75**, 211–218 (1970).
- Shimabukuro, F. I. *Solar Phys.* **23**, 169–177 (1972).
- Akabane, K., Nakajima, H., Ohki, K., Moriyama, F. & Miyaji, T. *Solar Phys.* **33**, 431–437 (1973).
- Shimabukuro, F. I. *Solar Phys.* **15**, 424–432 (1970).
- Nakajima, H. *et al. Publ. astr. Soc. Jap.* (in the press).
- Kaufmann, P. *et al. C. Cult. S. Paulo* **36**, 664 (1984).
- Kaufmann, P., Strauss, F. M., Schaal, R. E. & Laporte, C. *Solar Phys.* **78**, 389–399 (1982).
- Orwig, L. E., Frost, K. J. & Dennis, B. R. *Solar Phys.* **65**, 25–37 (1980).
- Hertzberg Institute of Astrophysics *Solar Activity Rep. May 1984* (NRC, Ottawa, 1984).
- Kiplinger, A. L., Dennis, B. R., Emslie, A. G., Frost, K. J. & Orwig, L. E. *Astrophys. J. Lett.* **265**, L99–L104 (1983).
- Brenner, N. in *Astrophysics. Part C Vol. 12* (ed. Meeks, M. L.) 284–295 (Academic, New York, 1976).
- Kaufmann, P., Strauss, F. M., Opher, R. & Laporte, C. *Astr. Astrophys.* **87**, 58–62 (1980).
- Kaufmann, P. *et al. Solar Phys.* **91**, 359–376 (1984).
- Takakura, T. *et al. Nature* **302**, 317–319 (1983).
- Cornell, M. E., Hurford, G. J., Kiplinger, A. L. & Dennis, B. R. *Astrophys. J.* **279**, 875–881 (1984).
- Takakura, T., Uchida, Y. & Kai, K. *Solar Phys.* **4**, 45–57 (1968).
- Dulk, G. A. & Marsh, K. A. *Astrophys. J.* **259**, 350–358 (1982).

Single organic monolayer imaging by electron microscopy

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A range of water-insoluble materials, when spread from a solvent onto an air–water interface, form monolayers, Langmuir films, which can be built up on a solid substrate to give well-defined multilayer coatings, Langmuir–Blodgett (L–B) films, resembling fatty-acid layers^{1–3}. These L–B films have aroused intense interest because of their potential applications as electrically-active materials⁴, optical elements⁵ and electron beam resists⁶, for which it would be very useful to know the structure of the monolayers. Although significant structural information has been derived from electron diffraction studies, an electron micrograph would be ideal; because of the fragility of conventional materials and their degradation in the electron beam, however, such imaging has not been achieved till now. (Electron micrographs of L–B films of the heavy-metal complex $\text{Th}[(\text{CF}_3\text{CO})_2\text{CH}]_4$ have been reported⁷, but the films were not single monolayers.) We present here the first electron micrographs of a single monolayer.

Classic L–B materials resemble the fatty acids in having a hydrophilic headgroup (which interacts strongly with the water surface) coupled to a hydrophobic tail (typically a long alkyl chain). The first L–B films of phthalocyanines were prepared⁸

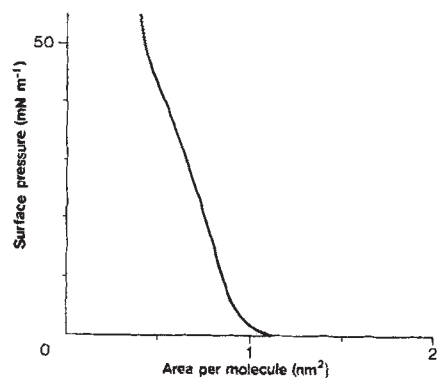


Fig. 1 Surface-pressure/area isotherm of compound (I).

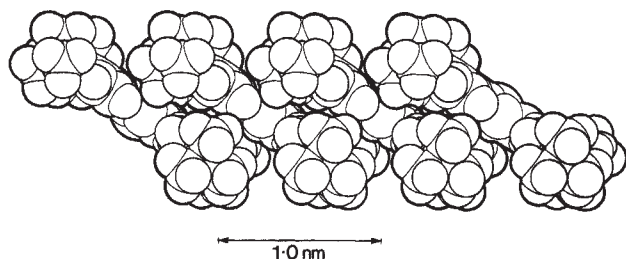


Fig. 2 Computer-generated model of a stack of (I) molecules.

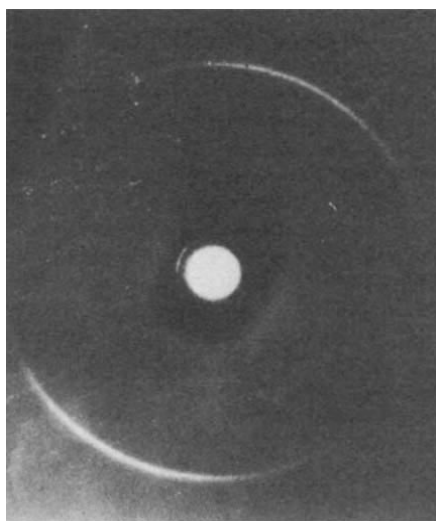
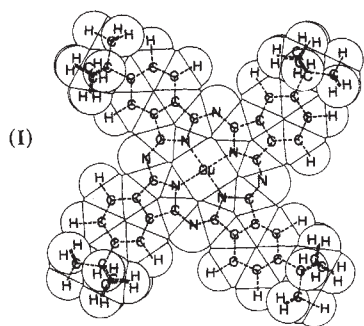


Fig. 3 Electron diffraction pattern of one monolayer of compound (I).

with multilayers on the water surface which were transferred to solid substrates. Hann *et al.*⁹ subsequently prepared and deposited monolayers of copper tetra-*t*-butylphthalocyanine (I).



Although the monolayer character of the films is consistent with the observed area per molecule of 0.96 nm^2 (Fig. 1), it is clear from the molecular structure that the packing cannot be dominated by the standard hydrophilic-hydrophobic interactions.

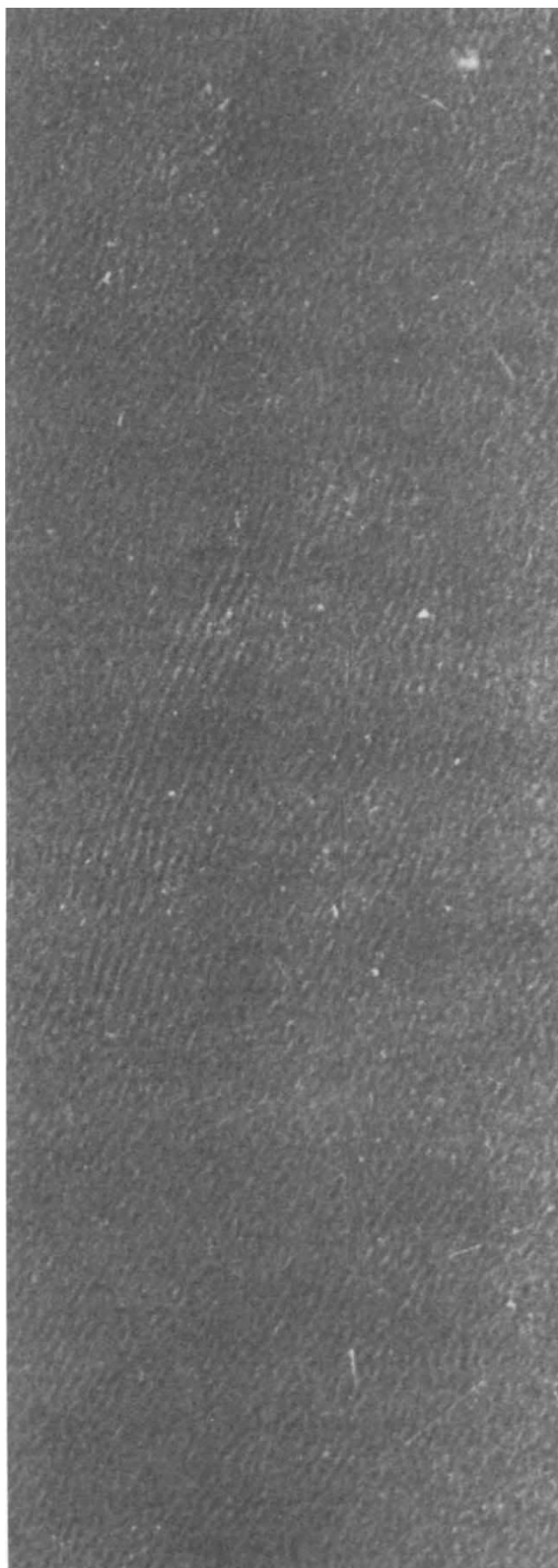


Fig. 4 Electron microscope image of one monolayer of compound (I).

Models indicate that the very bulky *t*-butyl groups dominate the packing, the molecules tending to become ordered in stacks in which the bulky groups are interleaved (Fig. 2). We therefore investigated whether the known stability of phthalocyanines^{10,11} in electron beams would allow direct electron microscope imaging of monolayers.

Monolayers of the compound (I) were spread at 20 °C from a solution in xylene (1 mg ml⁻¹) on to the surface of an ICI Langmuir trough^{5,12} whose barriers were compressed to obtain a surface pressure of 20 mN m⁻¹. The monolayer was then deposited on an electron microscope grid (coated with amorphous carbon) by lifting the horizontal grid through the monolayer.

The films supported on carbon were examined in a JEOL 1200EX electron microscope at 120 keV. Minimum dose conditions were used to obtain the maximum amount of structural information before the films degraded in the electron beam, but their lack of rigidity caused drift during photographic exposure—a major problem which could be overcome in future by using X-ray film.

The electron diffraction pattern (Fig. 3) has arced reflections corresponding to 1.9 nm and 0.33 nm, at right angles to one another, indicating that the molecules are lying in columns in the plane of the film with an intermolecular separation of 0.33 nm and an inter-columnar separation of 1.9 nm. We deduce also that the domains are small and highly disordered, yet the arcs

are sharp indicating good crystallographic order within the domains. The inter-columnar spacing measured from the electron diffraction pattern is in good agreement with the spacing calculated by molecular modelling.

A typical image is shown in Fig. 4. The lines of molecules are well ordered, but general bending occurs in the domain, highlighting one of the differences between electron diffraction and direct imaging; a severe bend in a domain would be represented in diffraction as two domains at an angle to each other. The contrast is in accordance with that expected from calculated images¹³ of unsubstituted phthalocyanines and the bending within each domain is comparable with that observed in copper phthalocyanine crystals that have undergone severe mechanical distortion¹⁴. Here the domains are only approximately aligned and those that are normally adjacent are not in contact, implying that the L-B film should be regarded as an amorphous matrix containing embedded crystallites.

The order in the film is significantly lower than that observed by electron diffraction of long-chain materials¹⁵ and the domains are much smaller than those observed optically in diacetylenes¹⁶. Hence we are presently studying how specific conditions of the preparation affect the ordering of phthalocyanine monolayers.

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1. Langmuir, I. *J. Am. chem. Soc.* **39**, 1848–1906 (1917).
2. Langmuir, I. *Trans. Faraday Soc.* **15**, 62–75 (1920).
3. Blodgett, K. B. *J. Am. chem. Soc.* **57**, 1007–1022 (1935).
4. Vincett, P. S. & Roberts, G. G. *Thin Solid Films* **68**, 135–171 (1980).
5. Pitt, C. W. & Walpita, L. M. *Thin Solid Films* **68**, 101–127 (1980).
6. Barraud, A. *Thin Solid Films* **99**, 317–321 (1983).
7. Baumeister, W. & Hahn, M. *Cytobiologie* **7**, 244–264 (1973).
8. Baker, S., Petty, M. C., Roberts, G. G. & Twigg, M. V. *Thin Solid Films* **99**, 53–59 (1983).

9. Hann, R. A. *et al. Proc. 2nd Int. Workshop Molecular Electron. Devices* (Washington, April 1983) (in the press).
10. Murata, Y., Fryer, J. R. & Baird, T. *Nature* **263**, 401–402 (1976).
11. Fryer, J. R. *Acta crystallogr.* **A35**, 327–332 (1979).
12. Roberts, G. G., McGinnity, T. M., Barlow, W. A. & Vincent, P. S. *Thin Solid Films* **68**, 223–232 (1980).
13. O'Keefe, M. A., Fryer, J. R. & Smith, D. J. *Acta crystallogr.* **A39**, 838–847 (1983).
14. Fryer, J. R. *J. Microsc.* **120**, 1–14 (1980).
15. Naegele, D., Lando, J. B. & Ringsdorf, H. *Macromolecules* **10**, 1339–1344 (1977).
16. Grunfeld, F. & Pitt, C. W. *Thin Solid Films* **99**, 249–255 (1983).

The molecular properties governing solubilities of organic nonelectrolytes in water

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Among the important problems in solutions chemistry, hitherto unresolved, is that of identifying and evaluating the solute and solvent molecular properties governing solubilities of organic nonelectrolytes in water. There are few subjects having such widespread ramifications. The treatment of solubility phenomena in terms of molecular properties of both solutes and solvents is made particularly explicit in the approach in which the endoergic formation of a solvent cavity suitable for the solute molecule is opposed, on insertion of the solute, by exoergic terms involving solute–solvent intermolecular interactions. Recently¹, we used this approach, together with our intermolecular-force parameterizations, called solvatochromic parameters^{2,3}, to correlate and predict octanol/water partition coefficients for non-hydrogen-bond donor aliphatic solutes. We now report linear solvation energy relationships which, we believe, represent a resolution of the problem for non-hydrogen bonding, hydrogen-bond acceptor and weak hydrogen-bond donor liquid aliphatic solutes.

For these types of solutes, the leading exoergic term was found to be that for the hydrogen bonding by the hydrogen-bond donor (HBD) solvents to the hydrogen-bond acceptor (HBA) solutes, being given in each solvent (octanol/water) by the linear

solvation energy relationship (LSER), $(\Delta G_s)_{HB} = C\alpha_1\beta_2$, where α_1 is the solvatochromic parameter scaling HBD acidity of the solvent, β_2 is the corresponding parameter scaling HBA basicity of the solute, C is a proportionality constant, and $(\Delta G_s)_{HB}$ is the hydrogen-bonding contribution to the free energy of solution. A secondary exoergic term, measuring the dipolarity effect, is given by $(\Delta G_s)_{DP} = B\pi_1^*\pi_2^*$, where π^* is the solvatochromic parameter measuring solute and solvent dipolarity/polarizability.

The solute parameter that determines the magnitude of the cavity term is $\bar{V}$, the molar volume, taken here as the molecular weight divided by the liquid density of the solute; the complementary solvent parameter is the square of the Hildebrand solubility parameter, δ_H^{-2} . Accordingly, the endoergic cavity term is given by $(\Delta G_s)_{Cav} = A(\delta_H^{-2})_1\bar{V}_2/100$. We use $\bar{V}/100$ so that the scale of the cavity term should be similar in magnitude to those for π^* , α and β , which makes the evaluation of the relative contributions of the various terms to $(\Delta G_s)_{Total}$ easier.

Combining the three terms, we find that free energy-dependent solubility properties, S_p , are given by equation (1), which, for solubility in a single solvent, or distribution between a pair of solvents, reduces to equation (2)

$$S_p = S_{p_0} + A(\delta_H^{-2})_1\bar{V}_2/100 + B\pi_1^*\pi_2^* + C\alpha_1\beta_2 \quad (1)$$

$$S_p = S_{p_0} + m\bar{V}_2/100 + s\pi_2^* + b\beta_2 \quad (2)$$

An earlier correlation of octanol/water partition coefficients using equation (2)¹, since expanded to include aromatic non-HBD solutes and some alkanols discussed in detail elsewhere⁵, is given by equation (3)

$$\log K_{ow} = (0.20 \pm 0.07) + (2.74 \pm 0.05)\bar{V}/100 - (0.92 \pm 0.08)\pi^* - (3.49 \pm 0.09)\beta \quad (3)$$

for which $n = 102$, $r = 0.989$, s.d. = 0.17.

We have now found that molar solubilities in water at 25 °C of liquid non-HBD aliphatic solutes, S_w , and an almost

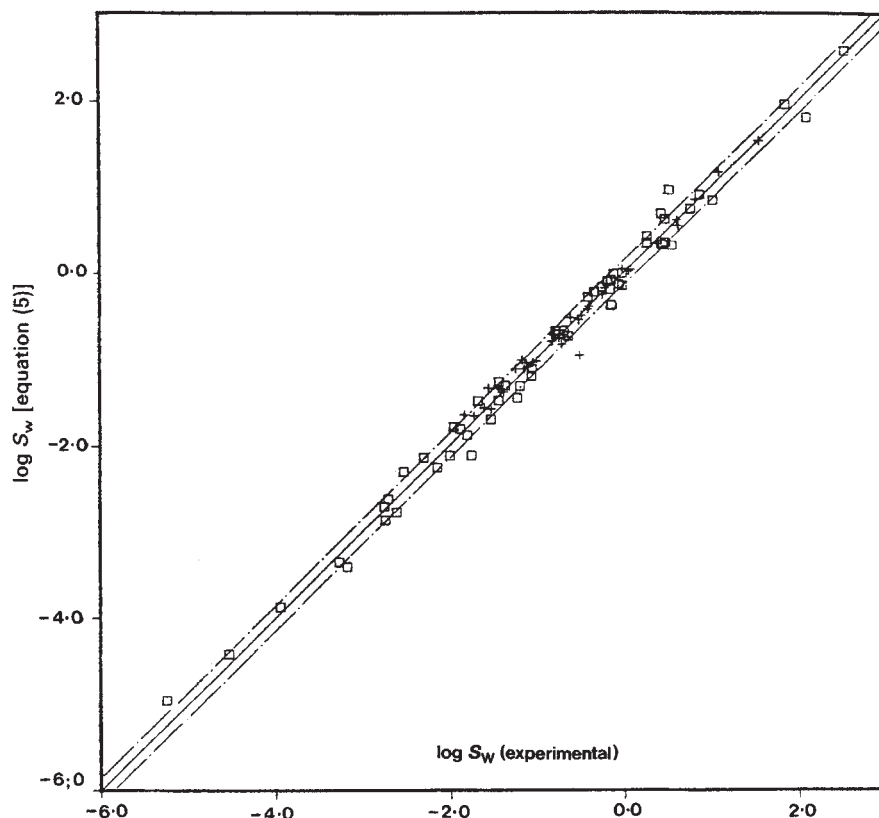


Fig. 1 Calculated (equation (5)) versus observed (experimental) water solubilities ($\log S_w$) of non-hydroxylic compounds, ($\square$) and alcohols (+) from the data given in Table 1.

Table 1 Solubilities in water and gas/water partition coefficients of liquid aliphatic nonelectrolytes at 25 °C

Solute	$\bar{V}/100$	π^*	β or β_m	$\log S_w$		$\log S_g$	$\log K_{gw}$		Uncertainty $\ddagger$
				Experimental*	Equation (5)		Experimental	Equation (5) $\dagger$	
<i>n</i> -C ₇ H ₁₆	1.465	-0.08	0.00	-4.53	-4.41				± 0.17
(C ₂ H ₅) ₃ N	1.401	0.14	0.71	-0.14	-0.38				± 0.25
CH ₃ COC ₂ H ₅	0.895	0.67	0.48	0.49	0.36				± 0.25
(C ₂ H ₅) ₂ O	1.046	0.27	0.47	-0.13	-0.38				± 0.22
C ₂ H ₅ COOCH ₃	0.963	0.55	0.45	-0.14	-0.08				± 0.24
C ₂ H ₅ NO ₂	0.721	0.82	0.25	-0.24	-0.19				± 0.23
HCON(CH ₃) ₂	0.770	0.88	0.69	(1.90)	1.98	-3.62	-5.52	-5.60	± 0.28
CH ₃ COCH ₃	0.734	0.71	0.48	(0.92)	0.92	-1.87	-2.79	-2.79	± 0.24
CH ₃ CON(CH ₃) ₂	0.924	0.88	0.76	(2.15)	1.83	-4.12	-6.27	-5.95	± 0.29
(CH ₃) ₂ SO	0.710	1.00	0.76	(2.59)	2.60	-4.62	-7.21	-7.22	± 0.29
CH ₃ COOC ₅ H ₁₁ - <i>n</i>	1.487	0.53	0.45	-1.74	-1.85	-3.61	-1.80	-1.76	± 0.27
CH ₃ OH	0.405	0.60	0.40	(1.35)	1.56	-2.21	-3.56	-3.77	± 0.20
(CH ₃) ₂ CHOH	0.765	0.48	0.51	(0.83)	0.87	-2.65	-3.48	-3.52	± 0.23
<i>n</i> -C ₇ H ₁₅ OH	1.414	0.43	0.45	-1.83	-1.65				± 0.25
2-C ₆ H ₁₃ OH	1.255	0.44	0.51	-0.82	-0.80				± 0.25
<i>t</i> -C ₅ H ₁₁ OH	1.094	0.41	0.57	0.09	0.04				± 0.24

* Values in parentheses are for $\log (S_g/K_{gw})$. Although these are 'hypothetical' solubilities for compounds that are fully miscible with water, multiplying them by 1.364 would give real free energies of solution in kcal mol⁻¹.

$\dagger$ Obtained by subtracting calculated values of $\log (S_g/K_{gw})$ from values of $\log S_g$ in the literature.

$\ddagger$ These are the 'worst case' combined uncertainties (that is, assuming all the $\pm$ values in equation (5) are either all plus or all minus) which show that there is a total threefold range of calculational uncertainty for a property that varies experimentally by seven orders of magnitude between *n*-heptane and dimethyl sulphoxide. The maximum uncertainty is about twice the standard deviation of equation (5).

equivalent quantity, S_g/K_{gw} (where S_g is the molar concentration in the solute saturated vapour ($S_g = P_{atm}/24.5$) and K_{gw} is the gas/water partition coefficient), are also well correlated by a multiple linear regression equation in the form of equation (2). The data are mainly those of Hine and Mukherjee⁶ and Amidon *et al.*⁷; the correlation is given by equation (4)

$$\begin{aligned} \log S_w &= \log (S_g/K_{gw}) \\ &= (0.52 \pm 0.12) - (3.33 \pm 0.09) \bar{V}/100 \\ &\quad - (0.47 \pm 0.11) \pi^* + (5.23 \pm 0.12) \beta \end{aligned} \quad (4)$$

for which $n = 56$, $r = 0.9948$, s.d. = 0.166.

Weak HBD alkanol solutes can apparently be included in equation (2) if we use the following β_m values (that is, β values for alcohols in the non-self-associated 'monomeric' form: methanol, 0.40; all primary alcohols, 0.45; all secondary alcohols, 0.51; all tertiary alcohols, 0.57. These are very close to the following β_m values determined by Abboud *et al.*⁸ from formation constants for the 1:1 complexes of the monomeric alcohols with 3,4-dinitrophenol in cyclohexane: methanol, 0.415; ethanol, 0.47; 2-propanol, 0.51; *t*-butanol, 0.56.

Using these new solvatochromic parameters for the alkanols, and combining the data of Yalkowsky and Valvani⁹ for 37 R-OH compounds (excluding the C₈ and higher alkanols; for reasons

which will be discussed elsewhere, the correlations better accommodate higher molecular weight solutes if a term in log vapour pressure is also included) with the 56 solubility results treated earlier, we obtain equation (5)

$$\begin{aligned}\log S_w &= \log (S_g / K_{gw}) \\ &= (0.55 \pm 0.09) - (3.36 \pm 0.06) \bar{V} / 100 \\ &\quad + (0.46 \pm 0.09) \pi^* + (5.23 \pm 0.09) (\beta \text{ or } \beta_m)\end{aligned}\quad (5)$$

for which $n = 93$, $r = 0.9943$, $s.d. = 0.144$.

Values of S_w and K_{gw} calculated from equation (5) and S_g values from the literature are compared in Table 1 with experimental results for some representative solutes. Equation (5) is plotted in Fig. 1.

The fact that β_m values in equation (5) accommodate the results for the unsubstituted alkanols suggests that, although amphiprotic, these R-OH compounds act only as HBA bases, not HBD acids, in aqueous solution. This seems reasonable if one compares the relevant solvatochromic parameters: for R-OH 'monomer' solute, $\alpha_m \approx 0.3$, $\beta_m = 0.40\text{--}0.57$; for water-solvent clusters, $\alpha = 1.17$, $\beta \approx 0.2$. For R-OH to act as a hydrogen-bond donor or acceptor in aqueous solution it must usually do so at the expense of a water molecule or cluster acting as donor or acceptor at the same site. As β is larger for R-OH than for $(H_2O)_n$, there is a free-energy advantage when R-OH acts as a base; as α is smaller for R-OH than for $(H_2O)_n$, there is a free-energy disadvantage when R-OH acts as an acid. It follows, therefore, that equation (5) should not accommodate solubility results for stronger HBD R-OH compounds; this is indeed the case. If we use our best estimates of 0.05 and 0.03, respectively, for β_m of trifluoroethanol and hexafluoroisopropanol, we obtain the following log (S_g / K_{gw}) results:

	Equation (5)	Experimental
CF ₃ CH ₂ OH	-1.27	0.80
(CF ₃) ₂ CHOH	-2.82	0.74

The latter results suggest that to include stronger HBD solutes in equation (5) requires also either a term in α_m or an amphiprotic hydrogen-bonding parameter.

As $\bar{V}/100$, π^* and β have been scaled to cover roughly the same range, it follows from equations (4) and (5) that the major factors influencing solubilities of organic nonelectrolytes in water are the exoergic term due to water donor-solute acceptor hydrogen-bonding, and the opposing endoergic cavity term, with solute-H₂O dipolar and induced dipolar interactions exerting only a second-order influence. Equations (4) and (5) are also important in that they confirm the widespread scope and applicability of the β scale of HBA basicities, and relate solubilities in water to a wide variety of physicochemical properties and reactivity parameters which have been correlated by β , either alone or in combination with the π^* , α and/or ξ parameters²; these include positions and intensities of maximal absorption in infrared, nuclear magnetic resonance, electron spin resonance and ultraviolet/visible absorption and fluorescence spectra; logarithms of reaction rate and equilibrium constants; free energies and enthalpies of formation of hydrogen-bonded and Lewis acid/base complexes; GLC and HPLC capacity factors and retention indices; adsorption properties on charcoal; and physiological and toxicological QSARs (quantitative structure/activity relationships).

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- Kamlet, M. J., Abraham, M. H., Doherty, R. M. & Taft, R. W. *J. Am. chem. Soc.* **106**, 464-466 (1984).
- Kamlet, M. J., Abboud, J.-L. M., Abraham, M. H. & Taft, R. W. *J. org. Chem.* **48**, 2877-2887 (1983).
- Kamlet, M. J., Abboud, J.-L. M. & Taft, R. W. *Prog. phys. org. Chem.* **13**, 485-630 (1981).
- Kamlet, M. J., Carr, P. W., Taft, R. W. & Abraham, M. H. *J. Am. chem. Soc.* **103**, 6062-6066 (1981).
- Taft, R. W., Abraham, M. H., Famini, G. R., Doherty, R. M. & Kamlet, M. J. *J. pharm. Sci.* (submitted).
- Hine, J. & Mookerjee, P. K. *J. org. Chem.* **40**, 292-298 (1975).
- Amidon, G. L., Yalkowsky, S. H., Anik, S. T. & Valvani, S. C. *J. phys. Chem.* **79**, 2239-2246 (1975).
- Abboud, J.-L. M., Sraidi, K., Guiheneuf, G., Kamlet, M. J. & Taft, R. W. *J. org. Chem.* (submitted).
- Yalkowsky, S. H. & Valvani, S. C. *J. pharm. Sci.* **69**, 912-922 (1980).

Deep structure of southern Tibet inferred from the dispersion of Rayleigh waves through a long-period seismic network

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Our understanding of the tectonic framework for the formation of Tibet and the collision of India with Eurasia¹ depends on our knowledge of the regional structure of the crust and upper mantle. Such basic information can be derived from seismic studies, but lateral inhomogeneities cause problems with interpretation of external observations, owing to the lack of stations inside a hardly accessible landmass. In 1982, as part of the French-Chinese cooperation programme, a network of long-period seismic stations was installed for the first time in southern Tibet itself with the object of obtaining local pure-path seismic data. Previously (with the exception of ref. 2) pure-path Tibet dispersion curves were extracted from mixed-path observations³⁻⁹, which required assumptions about neighbouring regions and focal mechanisms. In this study, local Rayleigh phase-velocity dispersions from the network were inverted to yield S-wave velocity models. As the period range of the data extends beyond 120 s, the information so obtained constrains deep-seated structures down to 200 km. The crust is shown to be very thick (65-70 km), in accordance with previous estimates²⁻⁹, and is divided into an upper part with a velocity of 3.2 km s⁻¹ and a lower part with a steep velocity gradient. The underlying mantle has a high velocity lid ($V_s = 4.7$ km s⁻¹), beneath which the structure resembles that of western Europe, with a relatively weak velocity minimum (compared with active tectonic regions) of 4.36 km s⁻¹ at a depth of 150 km.

The seismograms used here were recorded by four SL-210 Geotech vertical seismometers, with free periods made equivalent to 120 s by a feedback device^{10,11}. The seismometers are equipped with a triggered digital recorder with a sampling interval of 1 s. Great-circle-propagation paths for the four largest teleseismic events (Table 1) are almost in line with pairs of stations (Fig. 1); except for the Honshu event, the alignment defined by the theoretical azimuth, Az_1 , is slightly outside 10° of the great-circle path, but this is corrected by δAz after a multi-station analysis. For the Kuril Islands earthquake, two pairs of stations are in line; for the El Salvador earthquake a reverse profile was provided by R2 (see Table 1).

The two-station phase velocity measurements have been performed by several classical methods and the results for the profiles (1) and (4) are displayed in Fig. 2. On these dispersion curves for the Honshu and El Salvador earthquakes, convergent estimates were obtained for periods up to 150 s, but observations were limited to 70 s for the Kuril Islands earthquake.

For the Kuril Islands and Honshu earthquakes, using the records from all four stations, a wave-front field was constructed by a 4-stations analysis. For a tripartite network, phase velocities must be estimated from the two best-aligned stations, the third station giving the azimuth of arrival of the waves¹². The variation with frequency of the phases of the Fourier transforms of the records in the four stations were smoothed in the period range 24-57 s. Then, using a weighted least-squares method, an apparent azimuth, Az_c , (Table 1) and average phase velocity are estimated from which apparent local velocities for the two in-line stations are deduced. The results for the Kuril earthquake are very coherent, as the local dispersion does not differ from the average phase velocity by more than 0.01 km s⁻¹. This average phase velocity, curve (2+3) in Fig. 2, is very close to

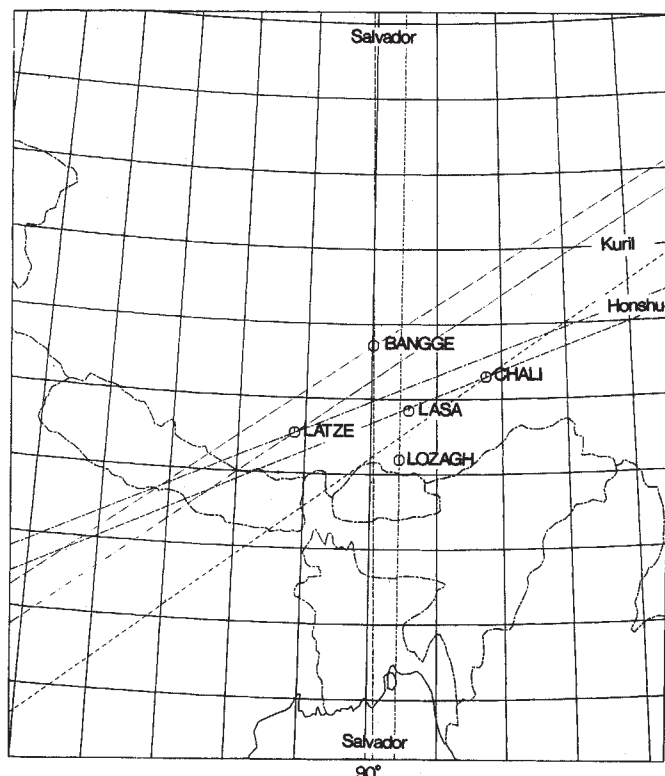


Fig. 1 Great-circle propagation paths for the four largest teleseismic events and the seismic stations at Bangge (90.04° long., 31.43° lat.), Chali (93.50°, 30.60°), Latze (87.68°, 29.08°) and Lozagh (90.87°, 28.39°).

the dispersion curve (4). For the Honshu earthquake, at periods shorter than 40 s, a rather strong deviation of azimuth, δA_z (Table 1) and negative local velocity anomaly are obtained at Latze, which could be owing to a perturbation during the propagation through the Nyainqentangliha zone southwest of Chali, as the western part of the path near Latze is quite similar to the path towards the Kuril Islands.

The slightly lower velocity of curve (1) (profile Chali-Latze) relative to (4) for periods < 70 s may be explained by the path going under grazing incidence along a heterogeneous transition zone between the southern and northern geological provinces¹³. Thus we will consider the curve (4) as representative of the phase velocity under the network and the reverse profile confirms the validity of our estimate of the error bar (± 0.015 km s⁻¹).

To interpret the data, two models were first examined (Fig. 3). Our best fit at short periods (Fig. 2) uses 70 km for the thickness of the crust and after consideration of the theoretical curves for 70-km and 80-km thickness and the error bar, this estimate becomes 70 ± 5 km. The velocities in the lid between 70 and 100 km have been taken as 8.4 km s⁻¹ for P waves and 4.65 km s⁻¹ for S waves, in agreement with P_n and S_n values¹⁴. Two models of the mantle below 100 km¹⁵ (Fig. 3) have been tried at long periods: the first one, EU, has the S-velocity stable

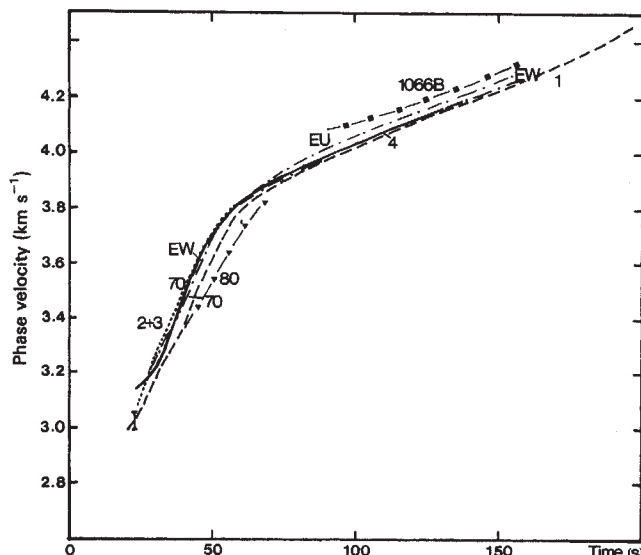


Fig. 2 Phase velocity data curves: (1), Honshu earthquake (Chali-Latze) and average velocity from a 4-station analysis; (2+3), Kuril Islands earthquake (average velocities from a 4-station analysis); (4), El Salvador earthquake (Bangge-Lozagh). The error bar, shown at short periods, is valid for all observations. Theoretical curves: the curves at short periods correspond to crustal thicknesses of 70 (—•) and 80 km (—▲); EU (—•) and EW (—•) are derived from European models¹⁵, replacing the first 100 km by a 70-km-thick crust, overlying a high-velocity mantle lid. The curve 1066B (—■) is obtained from a model by Gilbert and Dziewonski²².

structure inferred for paths across northern Eurasia, without a low-velocity layer, but with velocities a little smaller than for a shield; the second, EW, for western Europe, has a velocity minimum of 4.36 km s⁻¹ at 150-km depth and its upper mantle structure is very close to that of a continental Gutenberg model. Figure 2 shows the close agreement at long periods, between the Honshu (1) and Salvador (4) observations and the theoretical dispersion for EW.

To obtain a more refined model for the structure of the crust, a better fit than that for EW was sought at periods shorter than 60 s. The model EW, with a 70-km-thick crust, was used as a starting model in a least-squares inversion¹⁶. Two S-wave-velocity crustal models, with and without a discontinuity at a depth of 30 km, were considered. Indeed a discontinuity at that depth for P waves was recognized on some, but not all, explosion seismology profiles for southern Tibet¹⁷.

We inverted two series of data: the 'normal' dispersion curve (4) and the possibly-anomalous dispersion curve (1). The solutions obtained in the crust with and without a discontinuity are very similar (Fig. 3), the constraint of a discontinuity at 30-km depth introducing low-velocity layers. According to Fig. 3, these cannot be resolved by the data alone. A high velocity upper mantle lid is required by the data: starting from model EU, the high-velocity lid is apparent as early as the first iteration, but this solution is not shown in Fig. 3 as its fit to the data is much poorer than when starting from model EW.

Table 1

Earthquakes	Date	H_0 (h min s)	Lat.	Long.	M_s	H (km)	Station 1-Station 2	Profile no.	Δ_{12} (km)	Az_1	Δ_1 (km)	δA_z
El Salvador	19/6/82	06 21 58	13.31°N	89.34°W	$m_b 7.0$	52	{Bangge-Lozagh(R1)} {Lozagh-Bangge(R2)}	(4)	337.12	12.81° 13.58°	15069.15 24960.85	
Kuril Islands	30/6/82	01 57 34.1	44.68°N	151.14°E	6.9	21	{Chali-Lozagh} {Bangge-Latze}	(3) (2)	329.24 309.7	8.90° 14.34°	5208.24 5430.21	{3°(24s) 0°(40s) 6°(24s) 8°(40s) 0°(50s)}
Honshu	23/7/82	14 23 53.5	36.19°N	141.70°E	6.3	37	Chali-Latze	(1)	584.02	-5.97°	4481.34	

$\delta A_z = Az_1 - Az_c$, deviation from great-circle path; Az_1 is the theoretical azimuth, relative to the line of two stations, of the epicentre-station great-circle path at station 1; Az_c is an apparent azimuth corresponding to the wave-front field computed by a 4-station analysis. H_0 is the origin time of the earthquake and H is its depth. Δ_1 is the distance from the epicentre to station 1 and Δ_{12} is the distance between the stations 1 and 2.

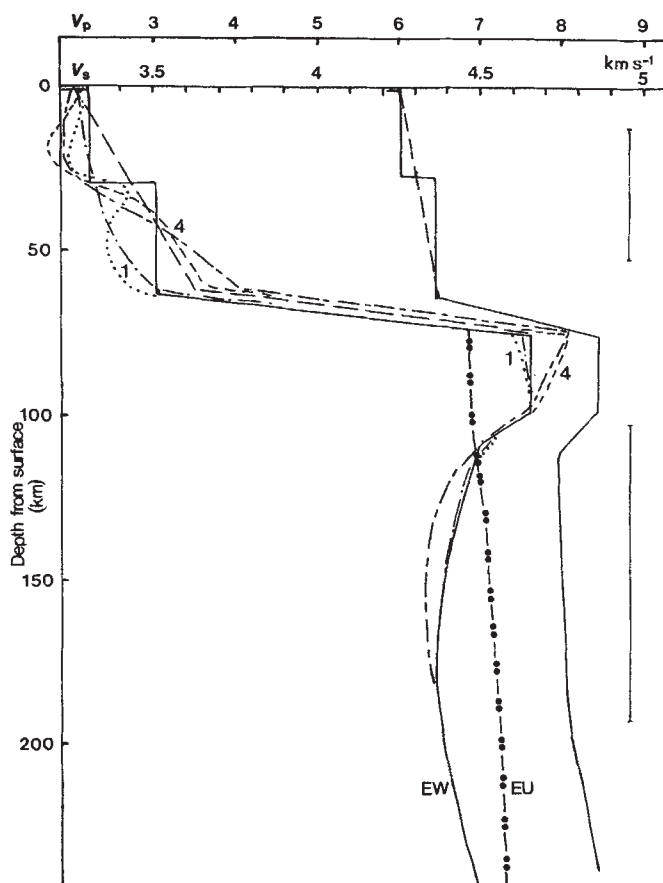


Fig. 3 Starting models: curves for model EW in the mantle, (—) and (---) differing only in the crust, are compared with a curve for model EU (---•---). Corresponding solutions starting from model EW (those for EU are not shown): S-wave-velocity solutions for the data (4) (---) and (---) and solutions for data (1) (---•---) and (---•---). The vertical bars on the right are the resolving widths at depths of 33 and 150 km.

For the normal dispersion data (4) one obtains a reduced crustal velocity in the first 30 km and a strong gradient with higher velocities in the lower half. For the anomalous dispersion data (1), the velocities are reduced mainly in the lower half of the crust, very slightly under the Moho, but not deeper, which is the effect of the anomalous zone (Fig. 1) perturbing the Rayleigh wave along a part of the path (1).

Thus, comparing the model obtained from the dispersion curve (4) with a continental model such as EW¹⁵, the main difference lies in the crust, which is twice as thick, has a lower average S-velocity and two different layers, the deepest one with an increased velocity. This crustal structure may be compared with an average of the explosion seismological results¹⁸ for P waves along a neighbouring profile. In some places a deeper reflector appears, beneath a shallow reflector of variable depth. Our results confirm an interpretation of a complex crust-mantle interaction between the two reflectors, which results in a mean crustal velocity higher in the lower than in the upper half.

The high-velocity lid is 30 km deeper than in a classical continental model, the mantle velocity decreasing with depth towards a slight velocity minimum (4.36 km s^{-1}) at a depth apparently unchanged relative to the continental model. Because of the lack of resolution, however, this depth is imprecise.

From our inversions, a model with a thick high-velocity lid is precluded by the data. Figure 2 shows that a model with a thick lid such as EU is ruled out, but at the limit of the error bar.

In our solution, if the thickness of the crust is twice that expected for a continental model, it does not seem that the high-velocity lid of the mantle is anomalously thick. Indeed a rather thin high-velocity lid is consistent with ref. 7, but not

with some of the conclusions of ref. 19. However, our mantle structure is closer to that of a stable continent^{20,21} than to that of a tectonic region.

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1. Molnar, P. & Tapponnier, P. *J. geophys. Res.* **85**, 5361-5375 (1978).
2. Romanowicz, B. *J. geophys. Res.* **87**, 6865-6883 (1982).
3. Patton, H. *Rev. Geophys. Space Phys.* **18**, 605-625 (1980).
4. Gupta, H. K. & Narain, H. *Bull. seismol. Soc. Am.* **57**, 235-248 (1967).
5. Chun, K. Y. & Yoshii, T. *Bull. seismol. Soc. Am.* **67**, 737-750 (1977).
6. Tung, J. P. & Teng, L. *EOS* **55**, 359 (1974).
7. Chen, W. P. & Molnar, P. *J. J. geophys. Res.* **86**, 5937-5961 (1981).
8. Yao, Zh. X. *et al. Acta geophys. sin.* **24**, 287-295 (1981).
9. Pines, I., Teng, T., Rosenthal, R. & Alexander, S. *J. geophys. Res.* **85**, 3829-3844 (1980).
10. Fels, J. F. *Notice technique de l'Institut de Physique du Globe* (Paris, 1977).
11. Souriau, A. thesis, Univ. Pierre et Marie Curie (1978).
12. Knopoff, L. *et al. Bull. seismol. Soc. Am.* **56**, 1009-1044 (1966).
13. Tapponnier, P. *et al. in Collision Tectonics* (eds Ries, A. & Coward, M.) (Geological Society of London, 1984).
14. Barazangi, M. & Ni, J. *Proc. Int. Un. Geodesy. Geophys. General Assembly* (Hamburg), 49 (1984).
15. Cara, M., Nercessian, A. & Nolet, G. *Geophys. J. R. astr. Soc.* **61**, 459-478 (1980).
16. Tarantola, A. & Valette, B. *Rev. Geophys. Space Phys.* **20**, 219-232 (1982).
17. Hirn, A. *et al. Nature* **307**, 23-25 (1984).
18. Hirn, A. *et al. Nature* **307**, 25-27 (1984).
19. Ni, J. & Barazangi, M. *Geophys. J. R. astr. Soc.* **72**, 665-689 (1983).
20. Knopoff, L. *Geophys. J. R. astr. Soc.* **74**, 55-81 (1983).
21. Feng, Ch. & Teng, T. L. *J. geophys. Res.* **88**, 2261-2272 (1983).
22. Gilbert, S. & Dziewonski, A. M. *Phil. Trans. R. Soc.* **278**, 187-269 (1975).

Strain trajectories above the Main Central Thrust (Himalaya) in southern Tibet

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As a result of the collision of India with Asia¹⁻³, a thick pile of Precambrian crystalline rocks (the Main Central Sheet) was transported southwards over the Indian continent. A marked linear fabric (a mineral-stretching lineation) within the Main Central Sheet varies only slightly from a north-south trend which is interpreted as paralleling the direction of transport of the nappe³⁻⁶. Our study sought to extend the previous observations to the hitherto-poorly-known part of the Main Central Sheet in southern Tibet (China). The stretching lineation is dominantly north-south in the lower part of the sheet, but exhibits continuous and discontinuous variations from north-south to east-west in the upper part⁷. These variations of lineation trajectory indicate two shear components, one subparallel to, and one at a high angle with, the Himalayan belt. The recognition of westward wrench shearing in the Main Central Sheet implies a component of relative displacement parallel to the Himalayan belt and not only a radial one as generally assumed in previous models. This suggests that the kinematic story of the intracontinental deformation following the continental collision is probably not one of simple north-south convergence.

The Lhasa-Khatmandu road, which runs along a deeply-eroded precipice, displays a spectacular section of the Main Central Sheet (Fig. 1). The uppermost rock unit comprises 3,000-4,000 m of dark metapelites interlayered with quartzites, calcschists and marbles, one to several tens of metres thick (Fig. 1A). They are penetrated and hornfelsed by lenticular

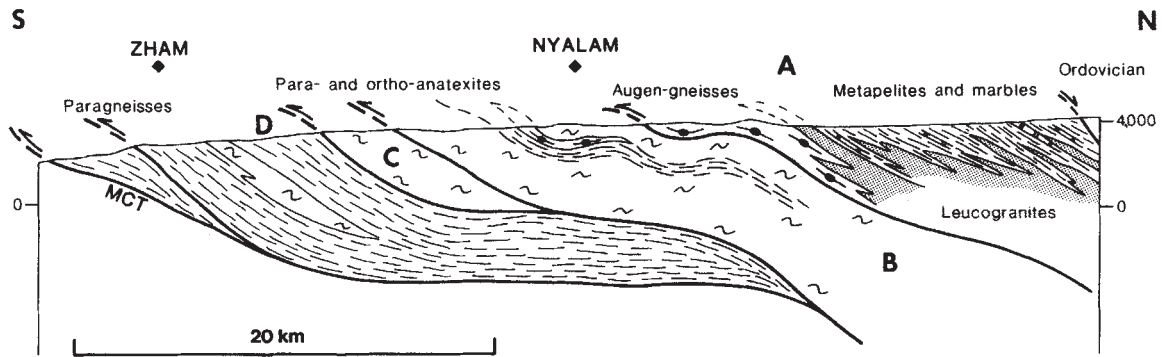


Fig. 1 Cross-section of the Main Central Sheet (Nyalam-Zham Valley).

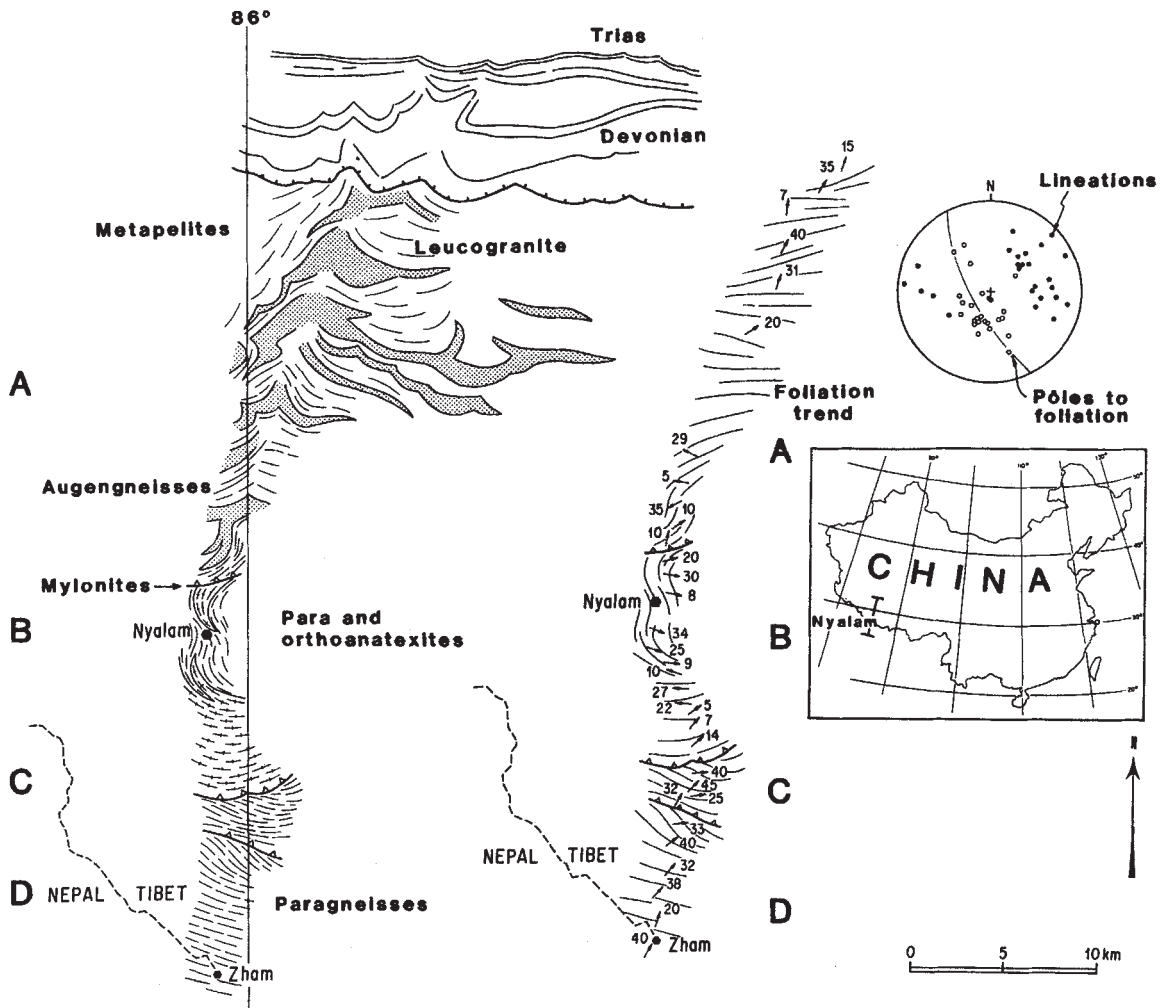


Fig. 2 Geological (left) and structural (right) maps along the Nyalam Zham Valley. The along section variations of foliation strike lines and stretching lineations attitude (arrows pointing in the plunge direction) are given on the structural map and on a stereo-plot (Wulff stereonet; lower hemisphere).

leucogranite plutons and large aplo-pegmatitic networks of dykes which lie parallel to the bedding or foliation plane of the country rocks. These plutons correspond to the Himalayan granites dated elsewhere of Eocene to Miocene age (52–17 Myr, refs 8–16) and show evidence of northwards shearing¹⁷. In the quartz plagioclase (An 30) biotite ± muscovite ± garnet epidote-metapelites, contact metamorphism has developed chloritoid, garnet and occasionally staurolite. Calcic layers are enriched in clinozoisite, hornblende, garnet and locally, scapolite. Marbles contain diopside and garnet within a calcite matrix. This rock unit is underlain by 1,000–2,000 m of homogeneous augen gneiss with K-feldspar porphyroblasts up to 15 cm in diameter. The augen gneisses could represent syntectonic granitoids although

similar rocks have provided early Palaeozoic ages in Nepal¹⁸. Below the augen gneisses 3,000–4,000 m of paraanateixites and orthoanateixites (Fig. 1B and C) are composed of quartz, plagioclase (An 25–30), K feldspar, biotite, muscovite ± garnet ± sillimanite ± cordierite ± hornblende ± clinozoisite ± diopside. They contain boudins of amphibolites composed of hornblende, plagioclase, epidote ± biotite ± garnet ± chlorite and sphene. Layers of quartzites and calcic gneisses can be found. Fine-grained pelitic layers contain, in addition to minerals observed in the anatexites, kyanite, garnet ± staurolite ± sillimanite. Some metres-thick mylonites occur parallel to the dominant foliation and are intruded by pegmatitic veins commonly folded into tight to isoclinal folds. Below the anatexites more or less anatectic

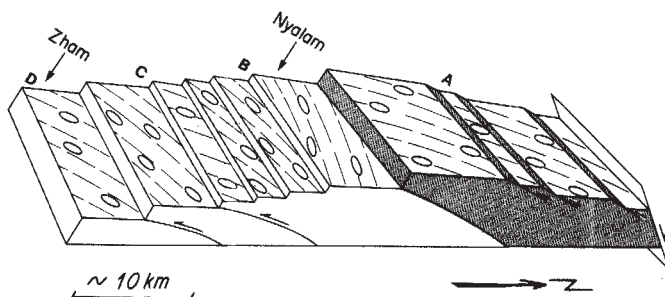


Fig. 3 Block diagram illustrating the structures and lineation trajectories in the Main Central Sheet. Shading shows metapelites intruded by Himalayan leucogranites with northward shearing microstructures.

and biotite-rich paragneisses contain euhedral kyanite, staurolite and garnet (Fig. 1D). They constitute the lowermost unit of the Main Central Sheet whose basis is the Main Central Thrust (MCT) itself¹⁹ (Fig. 1). In the whole pile, and especially in pelitic layers, the metamorphic minerals such as sillimanite, kyanite and micas appear to have crystallized or recrystallized during the deformation. This synmetamorphic character of the deformation is also demonstrated in migmatites by the localization of neosomes in shear bands, between boudins or in small-scale fold hinges.

The structure of the Main Central Sheet is simple. The general composite foliation dips 10–30° north, but is undulated, giving a large circular distribution with a pole oriented N060 on a stereoplot (Fig. 2). This distribution is due to long-wavelength low-amplitude folds in the vicinity of Nyalam (Fig. 1). The foliation is axial planar to numerous isoclinal recumbent folds which deform a foliation and which occur on the centimetre to several tens of metres scale. The folds and the foliations belong to a complex deformation history. It is difficult and even presumptuous to separate superposed phases known to result from continuous development of the Main Central Sheet. The isoclinal fold axes are parallel to a mineral lineation indicated by elongated patches of micas, kyanite and the long axes of sillimanite or muscovite + quartz aggregates. This lineation is also an elongation lineation which, because of the high values of shear strain within thrust sheets, can be equated with the direction of the principal stretch^{20,21}. Continuous and discontinuous variations in the lineation trend are related to the existence of several slices in the Main Central Sheet (Figs 1 and 2A, B, C and D). Although some discontinuities between the slices are lithological, some are intraformational. But they are all characterized by mylonitic bands and intense folding. In slice D, just above the MCT, the lineation trends N020–030 and rotates to N090–110° near slice C. Within slice C the same rotation is observed near slice B. In slices B and A, the mean direction of the lineation is N090 except to the base of these slices where it trends N020–030. The striking feature of the lineation trajectories (Figs 2 and 3) is the continuous anticlockwise rotation of the lineation when approaching the mylonites. Non-coaxial deformation criteria indicating shear deformation parallel to the lineation are abundant whether the lineation trends east-west or north-south. They are commonly sigmoid-shaped polycrystalline aggregates, asymmetric pressure shadows, rotated garnets, drag folds and shear bands, which are evidence for southwards shearing where the lineation is approximately north-south (in a way compatible with the sense of movement on the MCT) and westwards shearing where the lineation is almost east-west (a systematic trend which had not been observed previously).

In some places, channels 1–50 cm thick are parallel to the foliation. They correspond to phyllonites having a diaphoritic mineral composition (muscovite, pale chlorite) and along which post-metamorphic southwards inverse faulting has taken place.

They contain a specific east-west crenulation. To the bottom of the Main Central Sheet trending east-west are chevron folds several tens of centimetres in wavelength which are minor structures that are overturned to the south.

The Main Central Sheet in southern Tibet exhibits (particularly along the Nyalam–Zham section) the following characteristics: southwards thrusting suggested by the general dip to the north of the main composite foliation, north-south stretching lineation especially in mylonitic zones (implying that important north-south movements in the Main Central Sheet are concentrated within narrow zones) and southwards directed non-coaxial deformation criteria. In addition, westwards shearing subparallel to the Himalayan Belt is indicated by N080–120 trend of the principal extension and mineral lineation, west-directed non-coaxial deformation criteria, stability of the roughly east-west recumbent fold axes in the thrust pile where they should tend to be rotated into the shear direction²². East-west north-south lineations are observed within the same foliation plane and have developed with the same grade of metamorphism. The continuity between the two directions and the anticlockwise rotation of the lineation may be discussed in terms of combined (westward) wrenching and (southward) thrusting as it has been proposed for thrust areas in the Variscan Belt²³. This interpretation is new in the Main Central Sheet where earlier studies have ignored the east-west lineations and their significance. Our results indicate that the intracontinental thrust known as the MCT in the Himalayas has evolved into a ductile wrench fault. On one hand, the wrenching could be owing to local effects such as relative movement between slices induced by local mechanical heterogeneities; on the other hand it could be owing to general causes such as the inhibiting of underthrusting by the light buoyancy of the crust²⁴ or variations in the displacement path of India. To get an answer, more data are needed on the pattern of stretching trajectories and the geometry of thrust slices all along the Himalayan Belt. Some discontinuities not seen by us will probably be discovered in future work. The identification of mylonitic zones in the Main Central Sheet suggests that it is not a simple pile as claimed, but that it is the superposition of several tectonically emplaced slices whose nature and extension must be documented.

We emphasize that the evolution of large intracontinental thrust into wrench faults is probably a common feature of many more crustal shear zones than has been recognized.

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- Gansser, A. *Geology of the Himalayas* (Wiley Intersciences, London, 1964).
- Lefort, P. *Am. J. Sci.* **125A**, 1–45 (1975).
- Mattauer, M. *Earth planet. Sci. Lett.* **28**, 144–154 (1975).
- Pecher, A. thesis, Grenoble Univ. (1978).
- Bouchez, J. L. & Pecher, A. *Tectonophysics* **78**, 23–50 (1981).
- Brunel, M. thesis, Paris Univ. (1983).
- Brun, J.-P., Burg, J.-P. & Chen, G. M. *Terra Cognita* **3**, 246 (1983).
- Krummenacher, D. in *Recherches géologiques dans l'Himalaya du Nepal, Région de la Thakkhola*, (Ch. 6, 187–202 CNRS Paris, 1971).
- Chang, C. F., Zheng, X. L. & Pan, Y. S. *Proc. Inst. Geol. Acad. Sin. Peking*, 1–17 (1977).
- Vidal, Ph. *Geology* **6**, 196 (1978).
- Zhang, Y. Q., Dai, T. M. & Hong, A. S. *Proc. Symp. on Tibet Plateau*, **1**, 483–495 (1981).
- Xu, R. H., Sharer, U. & Allègre, C. J. *Terra Cognita* **3**, 273 (1983).
- Debon, F. *Terra Cognita* **3**, 266 (1983).
- Deniel, C. *Terra Cognita* **3**, 266 (1983).
- Ferrara, G., Lombardo, B. & Tonarini, S. *Geol. Rdsch.* **72**, 119–136 (1983).
- Scharer, U. *Earth planet. Sci. Lett.* **67**, 191–204 (1984).
- Burg, J.-P., Brunel, M., Gapais, D., Chen, G. M. & Liu, G. H. *J. struct. Geol.* (in the press).
- Le Fort, P. *9th Reun. Ann. Sci. Terre, Paris* **9**, 369 (1982).
- Shackleton, R. M. *J. struct. Geol.* **3**, 97–105 (1981).
- Nicolas, A. & Poirier, J. P. *Crystalline Plasticity and Solid State Flow in Metamorphic Rocks* (Wiley Intersciences, New-York, 1976).
- Hobbs, B. E., Means, W. D. & Williams, P. F. *An Outline of Structural Geology* (Wiley, New York 1976).
- Cobbold, P. R. & Quinquis, H. *J. Struct. Geol.* **2**, 119–126 (1980).
- Brun, J.-P. & Burg, J.-P. *Earth planet. Sci. Lett.* **61**, 319–332 (1982).
- Molnar, P. H. & Gray, D. *Geology* **7**, 58–62 (1979).

New estimates of asymmetric decomposition of racemic mixtures by natural β -radiation sources

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The Vester-Ulbricht hypothesis suggests that the chirality of biological molecules originates from the β -radiolysis of prebiotic racemic mixtures¹. Despite the inconclusiveness of past investigations²⁻⁴, recent calculations^{5,6} have shown that β particles, because of their helicity, radiolyse L- and D-enantiomers at slightly different rates, the asymmetry, A_R , being predicted to be 10^{-11} (new experimental tests^{7,8} give $|A_R| < 2 \times 10^{-9}$). Before this, the size of the radiolysis-induced chiral polarization, η_R ($\eta \equiv (n_L - n_D)/(n_L + n_D)$ where n_L and n_D are the numbers of L and D molecules present), was estimated⁹⁻¹² for different values of A_R ; according to Keszthelyi *et al.*⁹⁻¹¹, if $|A_R| \sim 10^{-11}$, $|\eta_R|$ can never exceed the chiral polarization, $|\eta_F|$, produced by statistical fluctuations, thus invalidating the V-U hypothesis. Here we re-examine the major assumptions on which these calculations were based and find that several overly restrictive conditions were imposed, which, when relaxed, allow the condition $|\eta_R| > |\eta_F|$, in accordance with the V-U hypothesis.

The previous calculations⁹⁻¹² of η_R were made for a particular system of chiral molecules undergoing simultaneous formation, β radiolysis, thermal- and radio-racemization and decomposition and, in some⁹⁻¹¹, well-defined physical restrictions were placed on the volume and surface area of the system. We follow here these earlier treatments, except that the restrictions are removed and three new features introduced: (1) the recent theoretical estimate $A_R \sim 10^{-11}$ is used; (2) a number of plausible new intense sources of β radiation are considered (Table 1);

Table 1 Source strengths and radiolysis rate constants for β -ray sources

β -ray source	Source strength $S(s^{-1} cm^{-3})$	Radiolysis rate constant $\epsilon S(s^{-1})$	Radiolysis time constant $(\epsilon S)^{-1} (yr)$
⁴⁰ K, ¹⁴ C in ocean	0.2	5×10^{-19}	6×10^{10}
Total crustal average	3.9	2×10^{-18}	2×10^{10}
Uranium-detrital deposit	6×10^2	4×10^{-16}	8×10^7
²⁶ Al	1×10^4	1×10^{-14}	3×10^6
²³⁵ U natural reactor	1.4×10^9	5×10^{-10}	60

The estimated source strengths and calculated values of the radiolysis rate constant and radiolysis time constant for several sources that may have occurred where the amino acids and sugars used in the formation of life were present. The values for the ocean and Earth's crust are taken from abundances of ⁴⁰K, ²³⁸U and ²³⁵U in ref. 18. In addition to these widespread sources, concentrated sources of radioactivity may have occurred in locations considered favourable to the formation of life—the most probable sources on the prebiotic earth being detrital uranium deposits (E. W. Heinrich, personal communication) occurring along beaches and in streams¹⁹. Characteristic source strengths for such deposits are taken from Hiemstra²⁰. In local areas, uranium concentrations as high as 10% can occur²⁰, possibly leading to the formation of natural nuclear reactors, such as at Oklo 2×10^9 yr ago²¹. The β intensity for a 10 kW reactor is taken from Lamarsh²². Finally, it has been speculated²³ that the amino acids present on the early Earth were formed in the presolar nebula and carried down in carbonaceous chondrites in the last stages of the Earth's accretion. These amino acids would have been irradiated during the decay of nova²⁴ or supernova²⁵ produced short-lived radioisotopes incorporated directly into the carbonaceous chondrites, for example, ²⁶Al, which has been detected in the Murchison and other meteorites²⁶. The ²⁶Al source strength is obtained from data in ref. 26.

(3) the temperature dependence of the racemization and decomposition rate constants is included.

The result of our calculation is that, for a system of N chiral molecules ($N = n_L + n_D$), $|\eta_R|$ increases with time from zero to a maximum value given approximately by

$$\eta_R(\max) \approx \begin{cases} \frac{1}{2} A_R & \text{if } \epsilon S \geq \beta \\ \epsilon S A_R / 2\beta & \text{if } \epsilon S \ll \beta \end{cases}$$

where ϵS is the radiolysis rate constant for a β -ray source of strength S (Table 1) and β is the racemization rate constant or half the thermal-decomposition rate constant, whichever is greater. This result is obtained by solving the rate equations¹² for η_L and η_D to first order in A_R . This maximum value is attained in a characteristic time (defined as the inverse of the respective rate constant) approximately equal to the radiolysis, racemization, or thermal decomposition time, whichever is least. These results are obtained irrespective of whether formation of the enantiomers is taken to occur before, or simultaneously with, radiolysis. For each of the β -ray sources, $|\eta_R(\max)|$ is plotted for the amino acid alanine as a function of temperature (Fig. 1) and the corresponding time required to reach this maximum is given for selected temperatures in Table 2. Because of uncertainties in the input parameters, these values are estimates of an order of magnitude rather than precise predictions.

We find that $|\eta_R|$ never exceeds 3×10^{-12} ; this is much smaller than previous estimates¹². Hence, asymmetric β -radiolysis can never by itself produce $|\eta| = 1$. Nevertheless, the small value of η_R can be amplified by subsequent chemical or biological processes eventually to give $\eta = \pm 1$. A necessary condition for the validity of the V-U hypothesis is that this initial η_R be greater in magnitude than $|\eta_F|$ and, because, from statistical arguments, $|\eta_F|$ is almost certainly $< 3N^{-1/2}$ (this equals three standard deviations, assuming the distribution of fluctuations to be gaussian), this condition will be satisfied for $N > 9/\eta_R^2$. For instance, if $\eta_R = 3 \times 10^{-12}$, then $N \sim 10^{24}$ (150 g of alanine) which, for reasons to be discussed below, may not be an excessively large amount.

Therefore, for plausible prebiotic conditions, the relation $|\eta_R| \geq |\eta_F|$ can obtain, whereas the most recent analysis of Keszthelyi¹³ concludes that $|\eta_R| < |\eta_F|$. The discrepancy arises because Keszthelyi's analysis is based on the choice of a system having a static 1-litre volume with a 1-cm² surface area and 10-m depth, which is radiolysed by β particles from natural ⁴⁰K at a temperature $T \approx 20^\circ C$ ^{9-11,13}, and assumes that the amino acids produced in the atmosphere enter this volume only through the surface, at a rate of $2 \times 10^{10} s^{-1} cm^{-2}$. According to the results

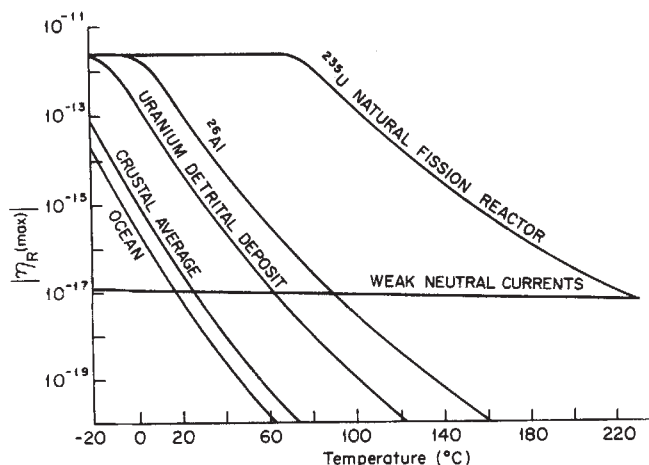


Fig. 1 Magnitude of maximum chiral polarization $|\eta_R(\max)|$ versus temperature for radiolysis of alanine in an aqueous environment by natural sources of β radiation. Calculations used the rate equations of Mann and Primakoff¹². Radiolysis rate constants, for which the asymmetry is 10^{-11} (refs 5, 6), are given in Table 1. The Arrhenius parameters from which the thermal racemization rate constants are calculated, the thermal decomposition rate constants and the radio-racemization rate constant were obtained from refs 27, 28 and 12, respectively. Clearly the largest values of $|\eta_R(\max)|$ are favoured by strong β emitters and by low temperatures (the temperature dependence of $|\eta_R(\max)|$ is a direct consequence of that of the racemization rate constant). Also shown for comparison is the chiral polarization produced by weak neutral currents, which create an energy difference between an enantiomer and its mirror image²⁹.

Table 2 Maximum chiral polarization times and magnitudes

β -Ray source	Temperature (°C)	Maximum chiral polarization $ \eta_R $ (max)	Time required (Yr)
^{40}K , ^{14}C in ocean	-20	2×10^{-14}	2×10^8
	0	3×10^{-16}	4×10^6
	20	7×10^{-18}	9×10^4
	100	1×10^{-22}	2
Total crustal average	-20	8×10^{-14}	2×10^8
	0	1×10^{-15}	4×10^6
	20	3×10^{-17}	9×10^4
	100	5×10^{-22}	2
Uranium-detrital deposit	-20	3×10^{-12}	8×10^7
	0	2×10^{-13}	4×10^6
	20	5×10^{-15}	9×10^4
	100	1×10^{-19}	2
^{26}Al	-20	3×10^{-12}	3×10^6
	0	3×10^{-12}	3×10^6
	20	1×10^{-13}	9×10^4
	100	3×10^{-18}	2
^{235}U reactor (10 kW)	-20	3×10^{-12}	60
	0	3×10^{-12}	60
	20	3×10^{-12}	60
	100	1×10^{-13}	2
	350	2×10^{-20}	2×10^{-7}

in Fig. 1, the largest value of $|\eta_R|$ that can be attained under these conditions is $\sim 10^{-17}$, requiring $N \geq 10^{35}$ for the condition $|\eta_F| \leq |\eta_R|$ to obtain. However, the number of amino acids accumulating in the system of Keszthelyi is, at most, of order 10^{27} (attained in 10^9 yr), which gives $|\eta_F| \approx 10^{-13}$ and $|\eta_F| > |\eta_R|$.

Figure 1 shows, however, that $|\eta_R| \geq |\eta_F| \approx 10^{-13}$ can obtain by increasing $|\eta_R|$ for the above system, either if $T < -20^\circ\text{C}$, or if the stronger sources that we have introduced are present. In addition $|\eta_F|$ can be smaller than 10^{-13} if larger systems are allowed, for example, by increasing the 1-cm^2 surface area of the systems in refs 9–11, 13. We note that subsequent amplification mechanisms may well impose limits on the size of a system, where 'system' is defined as the total number of chiral molecules, N , which is the starting point for chemical or biological processes amplifying $|\eta|$ in that initial population. However, the nature of such processes has not been determined explicitly, although models amplifying $|\eta|$ in systems of large physical dimensions have been demonstrated mathematically^{14–16}. Thus, we conclude that there is no justification for assuming $N < 10^{27}$ as in refs 9–11.

We have demonstrated that, under certain not improbable conditions, the result $|\eta_R| \geq |\eta_F|$ can obtain despite the small magnitude of η_R . Recently, a general argument that some specific causal mechanism probably dominated statistical fluctuations has been advanced¹⁷, which our results show could have been asymmetric radiolysis. Considering the present lack of knowledge of the details of any realistic prebiotic amplification process and evidence presented here for the prebiotic existence of the strong ^{235}U and ^{26}Al sources (Table 1), the V-U hypothesis cannot be excluded without further detailed research.

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- Vester, F., Ulbricht, T. L. V. & Krauss, H. *Naturwissenschaften* **46**, 68–69 (1959).
- Mason, S. F. *Nature* **311**, 19–23 (1984).
- Ulbricht, T. L. V. & Vester, F. *Tetrahedron* **18**, 629–637 (1962).
- Garay, A. S. *Nature* **219**, 338–340 (1968).
- Hegstrom, R. A. *Nature* **297**, 643–647 (1982).
- Zel'dovich, Ya. B. & Saakyan, D. B. *Sov. Phys. JETP* **51**, 1118–1120 (1980).
- Van House, J., Rich, A. & Zitzewitz, P. W. *Origins of Life* **14**, 413–420 (1984).
- Gidley, D. W., Rich, A., Van House, J. & Zitzewitz, P. W. *Nature* **297**, 639–643 (1982).
- Keszthelyi, L. *Origins of Life* **7**, 349–354 (1976).
- Fajsz, Cs. & Czege, J. *Origins of Life* **8**, 277–281 (1977).
- Czege, J., Fajsz, Cs. & Keszthelyi, L. *Origins of Life, Proc. 2nd ISSOL Meet. 5th ICOL Meet.* 333–378 (Center for Academic Publications, Japan Scientific Societies, Tokyo, 1978).
- Mann, A. K. & Primakoff, H. *Origins of Life* **11**, 255–265 (1981).
- Keszthelyi, L. *Origins of Life* **11**, 9–21, 191–194 (1981); **14**, 375–382 (1984).
- Hochstim, A. R. *Origins of Life* **6**, 317–366 (1975).
- Fajsz, Cs. & Czege, J. *Origins of Life* **11**, 143–162 (1981).

- Kondepudi, D. K. & Nelson, G. W. *Phys. Rev. Lett.* **50**, 1023–1026 (1983).
- Mann, A. K. & Primakoff, H. *Origins of Life* **13**, 113–118 (1983).
- Handbook of Chemistry and Physics* 52nd edn (1972).
- Heinrich, E. W. *Mineralogy and Geology of Radioactive Raw Materials* (McGraw-Hill, New York, 1958).
- Hiemstra, S. A. *Trans. geol. Soc. S. Afr.* **71**, 1–68 (1968).
- Kuroda, P. K. *Naturwissenschaften* **70**, 536–539 (1983).
- Lamarsh, J. R. *An Introduction to Nuclear Engineering* (Addison-Wesley, Reading, Massachusetts, 1975).
- Delsemme, A. H. *Origins of Life* **14**, 51–60 (1984).
- Clayton, D. D. *Astrophys. J.* **280**, 144–149 (1984).
- Cameron, A. G. W. & Truran, J. W. *Icarus* **30**, 447–461 (1967).
- Lee, T., Papanastassiou, D. A. & Wasserburg, G. J. *Geophys. Res. Lett.* **3**, 109–112 (1976).
- Williams, K. M. & Smith, G. G. *Origins of Life* **8**, 91–144 (1977).
- Miller, S. L. & Orgel, L. E. *The Origins of Life on the Earth* (Prentice-Hall, New Jersey, 1974).
- Hegstrom, R. A. *Origins of Life* **14**, 405–411 (1984).

Cyclic AMP-dependent protein kinase closes the serotonin-sensitive K^+ channels of *Aplysia* sensory neurones in cell-free membrane patches

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Selected actions of neurotransmitters and hormones on ion channels in nerve and muscle cells are now thought to be mediated by cyclic AMP-dependent protein phosphorylation^{1–11}. Although the cyclic AMP-dependent protein kinase (cAMP-PK) affects the cellular properties of several neurones, its mode of action at the single-channel level has not been characterized. In addition, little is known about the identity or subcellular localization of the phosphoproteins that control channel activity and, in particular, whether the critical substrate proteins are cytoplasmic or membrane-associated. In *Aplysia* sensory neurones, serotonin produces a slow modulatory synaptic potential mediated by cAMP-PK¹² that contributes to presynaptic facilitation and behavioural sensitization¹². Previously, we have found that serotonin acts on cell-attached membrane patches to produce prolonged all-or-none closures of a specific class of K^+ channels (S channels) whose gating is weakly dependent on voltage and independent of intracellular calcium^{13–15}. We demonstrate here that in cell-free membrane patches from *Aplysia* sensory neurones, the purified catalytic subunit of cAMP-PK¹⁶ produces all-or-none closures of the S channel, simulating most (but not all) aspects of the action of serotonin on cell-attached patches. This result suggests that protein kinase acts on the internal surface of the membrane to phosphorylate either the channel itself or a membrane-associated protein that regulates channel activity.

Single-channel currents were recorded from cell-free inside-out patches of membrane (cytoplasmic surface of the membrane facing the bath) from LE sensory neurones of *Aplysia* abdominal ganglia using standard techniques^{13,17}. The membrane potential across the patch was held at depolarized levels to inactivate both the delayed rectifier K^+ channel and the early K^+ channel¹⁸. In these conditions, the two types of channel currents observed in the cell-free patches corresponded to the non-inactivating S-channel current (I_s) and a Ca^{2+} -activated K^+ -channel current (I_{Ca})^{18,19}. The S channel in cell-free patches displays properties similar to those seen in voltage-clamp¹⁴ and cell-attached patch-clamp experiments^{13,15}. To study the action of cAMP-PK on S-channel currents in isolation, we kept the concentration of Ca^{2+} in the bath at $0.2 \mu\text{M}$, a concentration which does not activate the Ca^{2+} -activated K^+ channel in *Aplysia* at the potential at which the patch was held.

In experiments with cAMP-PK, S-channel currents were first identified in the inside-out patch on the basis of their Ca^{2+} -insensitivity and unit current amplitude. The patch pipette was

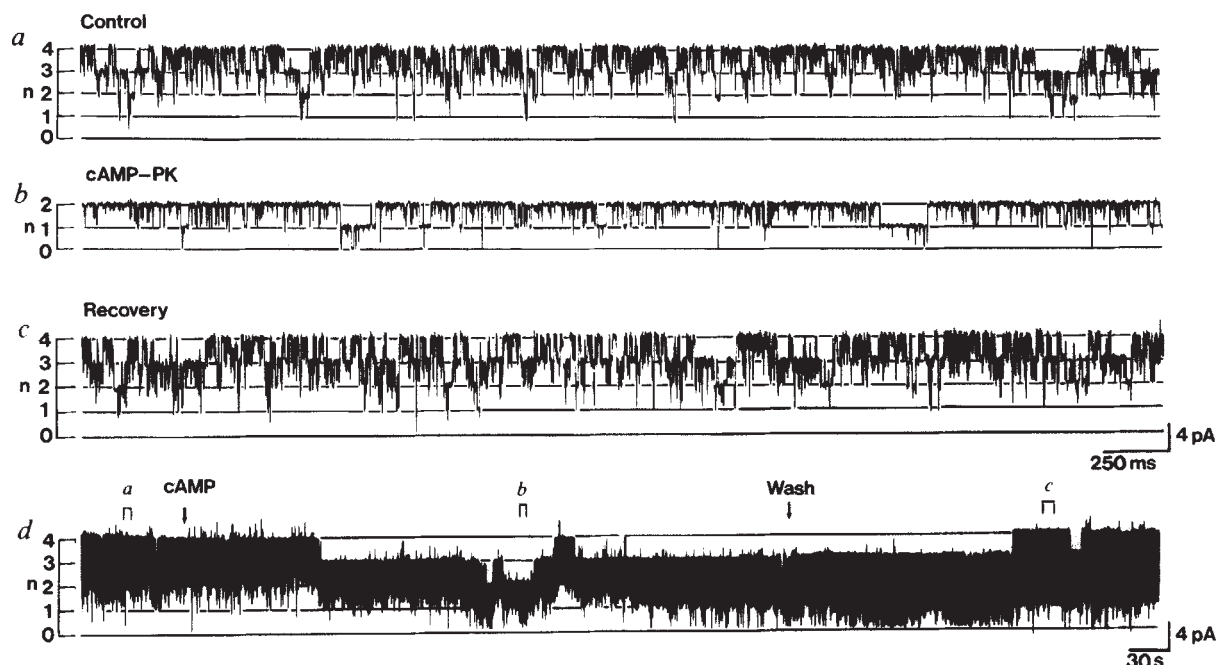


Fig. 1 cAMP-PK closes S channels in inside-out membrane patches from *Aplysia* sensory neurones. *a-c*, Expanded record of channel currents before, during and after application of cAMP-PK, respectively. 1 mM Mg-ATP was present initially. Ordinate shows the number of open channels (*n*). *a*, Before the addition of kinase, the patch contained four active channels open for a large fraction of the time. The current record fluctuates from one to four channels open simultaneously due to rapid random opening and closing of individual channels. The patch contained a fifth channel which opened occasionally throughout the experiment (seen as brief upward spikes in *d*) and which was not analysed further. *b*, Three minutes after addition of cAMP-PK (0.1 μ M final concentration) plus 1 mM Mg-ATP (2.0 mM final concentration) the current level fluctuates from zero to two channels open but the amplitude and probability of opening of the remaining active channels are unchanged from those of the control. *c*, After recovery, the channels again fluctuate between four levels. *d*, Closure and re-opening of the same channels shown in *a-c*, but on a slower time sweep. Channel currents were recorded for over 8 min before addition of cAMP-PK, during which time there were no closures longer than 10 s. At the arrow, cAMP-PK was added to the bath. After a latency of 3 min, one channel closed (downward step in maximal current level), and after a further 4 min, a second channel closed. The two closed channels then re-opened (upward step changes in maximal current level in *d*), followed by a final prolonged channel closure. On wash-out of the enzyme, the remaining closed channel re-opened after 5 min. The mean duration of cAMP-PK-produced closures in this experiment was 3 min. The expanded records were taken from the regions indicated by the brackets labelled *a*, *b* and *c* in *d*. In tabulating this experiment (Table 1), two out of five active channels were counted as closed by cAMP-PK. Probability of channel opening fluctuated between 0.5 and 0.7 throughout the experiment; these fluctuations were uncorrelated with the addition of kinase or Mg-ATP. Current records were filtered at 500 Hz in *d*, 1 kHz in *a-c*. The membrane was held at 0 mV throughout.

Methods. Patch pipettes were filled with normal artificial seawater (resistance 1–2 M Ω) containing (in mM): 460 NaCl, 10 KCl, 10 CaCl₂, 55 MgCl₂, 10 Tris buffer titrated to pH 7.5 with NaOH. The bath contained an 'intracellular' solution of (in mM): 360 KCl, 272 sucrose, 10 NaCl, 2 MgCl₂, 20 K-HEPES (pH titrated to 7.2–7.5 with KOH) and 1.5 K-EGTA and 1.0 CaCl₂ to give a free Ca²⁺ concentration of 0.2 μ M. All experiments were done at room temperature (22–25 °C). cAMP-PK was purified according to ref. 16 (specific activity 4.8 $\times 10^6$ U per mg protein where 1 U = 1 pmol PO₄³⁻ transferred per min, assayed according to ref. 25). Purified enzyme was stored in buffer containing 2 M glycerol, 50 mM KH₂PO₄, pH 7.2; 1–7 days before use, an aliquot (50–500 μ l) of kinase was dialysed against the 'intracellular' solution to remove the glycerol and raise the K⁺ concentration.

then placed in a small well, superfused with a solution containing 0.2 μ M free Ca²⁺ and channel currents were recorded for 5–15 min to obtain a stable baseline of S-channel activity. Purified catalytic subunit of cAMP-PK (0.1–1 μ M) was then added to the patch either with or without Mg-ATP.

In the presence of Mg-ATP, cAMP-PK produced prolonged all-or-none periods of channel closure (Fig. 1) in 73% of all experiments (*n* = 42). Such prolonged closures (lasting for over 20 s; see Fig. 2 legend) were observed neither during control recordings before the addition of kinase nor after the kinase was washed out of the bath. Once closed by the kinase, the channels do not remain closed indefinitely, but rather can re-open, even in the continued presence of kinase; there can then follow repeated cycles of long closures and re-openings.

The protein kinase closed 1.3 ± 1.0 (mean $\pm$ s.d.) channels out of a mean of 3.8 ± 1.9 channels initially active per patch. Thus, the kinase closed 34% of all channels in the patches, including channels in patches where kinase failed to produce any significant closure. To control for any nonspecific effects of the catalytic subunit of cAMP-PK not mediated by phosphorylation (as well as any bias introduced by our criteria for significant closure), we tested the effects of the catalytic subunit of cAMP-

PK in the absence of Mg-ATP. Without this source of high-energy phosphate, the kinase was much less effective, producing channel closures in less than 24% of all experiments and closing only 8% of all channels. Similarly, Mg-ATP alone (in concentrations up to 10 mM) had little effect on S-channel activity (see Fig. 2). We have not yet been able to determine whether the closures seen with cAMP-PK in the absence of Mg-ATP or with Mg-ATP alone reflect a low spontaneous closure rate of the channel that is independent of either the cyclic AMP-dependent kinase or Mg-ATP, or whether the closures result from the action of endogenous kinase or the presence of membrane-bound Mg-ATP. Nevertheless, the marked stimulation of channel closure by cAMP-PK in the presence of Mg-ATP argues that these closures represent a phosphorylation reaction mediated by the kinase.

The mean latency between addition of cAMP-PK and the first channel closure was 4.5 ± 4.0 min (range 30 s to 15 min). This latency was probably due partly to inadequate mixing when the small volume of enzyme (40 μ l) was added to the well. In the 10 most recent experiments, in which care was taken to ensure adequate mixing of the bath, the mean latency was reduced to <2.5 min.

We have compared our results in cell-free patches with the effects of serotonin on channels in intact cells. Extracellular application of serotonin and intracellular injection of cyclic AMP produce prolonged, all-or-none closures of the S channel without affecting either the elementary conductance of the channel or its gating¹³. It is important, therefore, that in cell-free patches, cAMP-PK simulates these physiological effects by

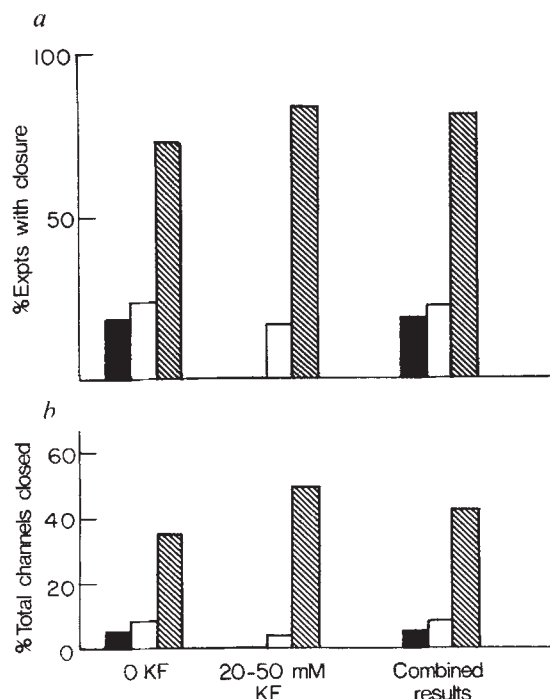


Fig. 2 Effects of Mg-ATP alone (■), cAMP-PK (□) and cAMP-PK plus Mg-ATP (▨) on S-channel currents. Data were obtained in the presence and absence of potassium fluoride as indicated. *a*, Per cent of experiments in which a given treatment produced prolonged closures of the S channel. Concentrations of cAMP-PK were in the range 0.1–1.0 μ M. The data have been grouped according to whether treatments included no KF (0 KF) or 20–50 mM KF in the bath solution. The pooled results from both sets of experiments are shown also. In tabulating the combined results, only results from treatments in the presence of fluoride were used whenever a given treatment was applied to a patch in both the absence and presence of fluoride. *b*, Per cent of total channels closed by the same treatments as in *a*. The increase in percentage of channels closed by cAMP-PK plus Mg-ATP with KF is statistically significant ($P < 0.05$). The percentage was obtained by dividing the maximum number of channels closed simultaneously by a given agent by the number of channels that were initially active in the patch. In determining the percentage of experiments with prolonged closure and the percentage of channels closed, a 20-s closure was chosen as the threshold for considering a prolonged closure significant, because this duration was generally longer than the longest closed time in the absence of agent but shorter than most of the prolonged closures seen in the presence of cAMP-PK (plus Mg-ATP). Similar results were obtained using a threshold of between 10 and 20 s. In addition, a closure was considered significant only if the baseline current recording in the absence of treatment was stable, with no closures longer than 20 s over a period of recording that was at least twice the latency to the first channel closure after addition of the agent. If a channel re-opened after 20 s, it was still counted as a closure. We did not calculate the average reduction in patch current, which would be somewhat less than the percentage of channels closed. The number of experiments using Mg-ATP, cAMP-PK and cAMP-PK plus Mg-ATP was, respectively, 26, 17 and 40 in 0 KF; 2, 6 and 12 in the presence of KF; and 27, 18 and 42 for the combined results. In addition to the control experiments shown here, we found that heat-inactivated cAMP-PK never produced the all-or-none closures of channels produced by the active kinase ($n = 8$), but in some experiments the denatured enzyme caused a slight decrease in the probability of opening of a channel. Such changes in probability were not seen with active kinase, either in the presence or absence of ATP.

closing channels in a prolonged, all-or-none way without also affecting conductance or gating. We observed two important differences, however, between the action of cAMP-PK in isolated patches and of serotonin in the intact cell. First, although in 25% of the experiments cAMP-PK produced closures that persisted for over 10 min, in most experiments the channels closed for a mean duration of 2–3 min then re-opened, even in the presence of the kinase, whereas serotonin caused closures that persisted for the entire duration of transmitter application (> 5 min). Second, cAMP-PK closed fewer channels than serotonin (34% as opposed to 46%; see Table 1 and Fig. 2).

One possible explanation of these differences is that the cell-free patch contains an endogenous protein phosphatase that limits the action of the kinase by cleaving the phosphate from the phosphoprotein²⁰; the lack of any specific phosphatase inhibitor for molluscan cells prevents us from testing this hypothesis directly. However, evidence consistent with the presence of phosphatase comes from two sources: (1) Preliminary experiments show that the membrane-cytoskeleton complex isolated from *Aplysia* ganglia²¹ contains significant phosphoprotein phosphatase activity (M.J.S. *et al.*, unpublished observations). This activity is largely inhibited by KF, a nonspecific phosphatase inhibitor²². (2) Based on these findings, we added KF to the solution bathing the inside of the patch, and found that KF increased both the percentage of successful experiments and the fraction of channels closed (Fig. 2). Figure 3 shows an example of such an experiment in which cAMP-PK initially produced no channel closure, but caused pronounced closures in the presence of fluoride. In three experiments in which kinase produced only brief closures (< 20 s long) in the absence of phosphatase inhibitor, the duration of channel closure was increased 5–10-fold in the presence of inhibitor (two experiments with fluoride and one with a different inhibitor, 2 mM *p*-nitrophenylphosphate). KF alone had no effect on channel activity nor did it potentiate either the effects of cAMP-PK in the absence of Mg-ATP or the effects of Mg-ATP alone (Fig. 2). As fluoride stimulates adenylate cyclase²³, we controlled for this by adding cyclic AMP directly to the patch to test whether the effects of KF are mediated by cyclic AMP production. In the presence of Mg-ATP, but without cAMP-PK, cyclic AMP (0.1–1 mM) had no significant effect on channel activity ($n = 6$), nor did it potentiate significantly the effects of cAMP-PK.

The results presented here indicate that cAMP-PK can produce prolonged all-or-none closures of single S channels in cell-free patches of membrane. The similarities in the actions of cAMP-PK in cell-free patches to those of serotonin and cyclic AMP acting through a complicated cellular system support the view that the closures produced by kinase in the cell-free patches are physiologically relevant. Moreover, these actions are produced by concentrations of enzyme (0.1–1 μ M) that are in the physiological range and represent less than the total concentration of cAMP-PK in the sensory neurones²⁴. The effects of

Table 1 Comparison of modulatory effects in cell-attached and cell-free membrane patches

Treatment	<i>n</i>	% Expts with closures	% Of channels closed	Latency to first closure (min)	Closed time (min)
Cell-attached					
Serotonin	33	76.5	45.8	1.7 ± 1.3	> 5
Cyclic AMP	7	71.4	52.2	4.5 ± 1.6	> 5
Cell-free					
cAMP-PK + ATP*	42	81.0	42.0	4.5 ± 4.6	3.0 ± 2.7

Closed times in serotonin and cyclic AMP generally outlasted the experiment so 5 min is a lower limit. Mean closed time with cAMP-PK was calculated using data from experiments in which channels showed recovery from closure ($n = 18$).

* Results are combined ($\pm$) fluoride results from Fig. 2.

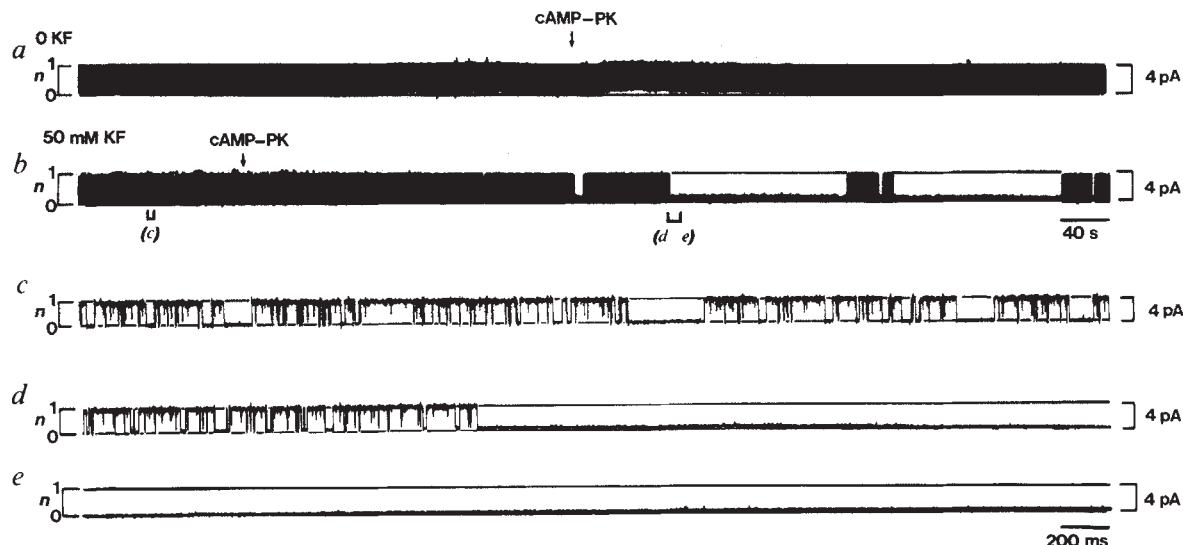


Fig. 3 KF potentiates the action of cAMP-PK. Current from an inside-out patch of membrane containing one S channel. *a*, Before the addition of 50 mM KF to the bath solution, application of cAMP-PK ($0.1 \mu\text{M}$; specific activity = 5.9×10^6 U per mg protein) and Mg-ATP (1.0 mM) had no effect on channel activity measured for 11 min after kinase application. *b*, Single-channel current recorded in the presence of 50 mM KF. Addition of cAMP-PK ($0.1 \mu\text{M}$) and Mg-ATP (1.0 mM) produced prolonged periods of channel closure (mean closed time = 2 min). *c*, Expanded time record (bracket labelled *c* in *b*) of single-channel current showing the normal channel gating process in the presence of 50 mM KF before the addition of kinase and Mg-ATP to the bath. *d*, *e*, Continuous sweeps on an expanded time scale showing the transition from the normal gating process to a period of prolonged closure after the addition of kinase plus ATP to the bath. Note that the channel exhibits normal gating behaviour up to the time at which it enters a prolonged closed state. Current record filtered at 250 Hz in *a*, *b* and 1 kHz in *c*–*e*.

cAMP-PK do not completely mimic those of serotonin and cyclic AMP in the intact cell, however. In the cell-free patch the kinase tends to close somewhat fewer channels and the closures are shorter, suggesting that either the cell-free patches lack adequate amounts of some component necessary for complete channel modulation, or the patches contain a phosphoprotein phosphatase which terminates the kinase-induced closures. That KF, a nonspecific phosphatase inhibitor, potentiates the effects of cAMP-PK provides indirect evidence for the involvement of a phosphatase. The difference in channel closure time between cell-attached and cell-free patches may be explained if the sensory neurones contain a cytoplasmic protein, similar to phosphatase inhibitor protein found in other tissues, which is capable of regulating phosphatase activity¹⁰. Finally, although we cannot determine whether the kinase phosphorylates the S channel itself, our results suggest that the isolated membrane contains at least some of the phosphoproteins necessary for channel closure.

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- Castellucci, V. F. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **77**, 7492–7496 (1980).
- Castellucci, V. F., Nairn, A. C., Greengard, P., Schwartz, J. H. & Kandel, E. R. *J. Neurosci.* **2**, 1673–1681 (1982).
- Kaczmarek, L. K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **77**, 7487–7491 (1980).
- Adams, W. B. & Levitan, I. B. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3877–3880 (1982).
- De Peyer, J. E., Cachelin, A. B., Levitan, I. B. & Reuter, H. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4207–4211 (1982).
- Doroshenko, P. A., Kostyuk, P. G., Martynuk, A. E., Kursky, M. D. & Vorobetz, Z. D. *Neuroscience* **11**, 263–267 (1983).
- Alkon, D. L., Acosta-Urquidí, J., Olds, J., Kuzma, G. & Neary, J. T. *Science* **219**, 303–306 (1983).
- Brum, G., Flockerzi, V., Hofman, F., Osterrieder, W. & Trautwein, W. *Pflügers Arch. ges. Physiol.* **398**, 147–154 (1983).
- Kennedy, M. A. *Rev. Neurosci.* **6**, 493–525 (1983).
- Nestler, E. J. & Greengard, P. *Nature* **305**, 583–588 (1983).
- Siegelbaum, S. A. & Tsien, R. W. *Trends Neurosci.* **6**, 307–313 (1983).
- Kandel, E. R. & Schwartz, J. H. *Science* **218**, 433–442 (1982).
- Siegelbaum, S. A., Camardo, J. S. & Kandel, E. R. *Nature* **299**, 413–417 (1982).
- Klein, M., Camardo, J. S. & Kandel, E. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5713–5717 (1982).
- Camardo, J. S., Shuster, M. J., Siegelbaum, S. A. & Kandel, E. R. *Cold Spring Harb. Symp. quant. Biol.* **48**, 213–220 (1983).
- Beavo, J. A., Bechtel, P. J. & Krebs, E. G. *Meth. Enzym.* **38**, 299–308 (1974).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Adams, D. J., Smith, S. J. & Thompson, S. H. A. *Rev. Neurosci.* **3**, 141–163 (1980).
- Meech, R. W. A. *Rev. Biophys. Bioengng* **7**, 1–18 (1978).
- Ingebritsen, T. S. & Cohen, P. *Science* **221**, 331–337 (1983).
- Saitoh, T. & Schwartz, J. H. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6708–6712 (1983).
- Revel, H. R. *Meth. Enzym.* **6**, 211–214 (1963).
- Rall, W. T. & Sutherland, E. W. *J. biol. Chem.* **232**, 1065–1076 (1958).
- Bernier, L. thesis, Univ. Columbia (1984).
- Maller, J. L., Kemp, B. E. & Krebs, E. G. *Proc. natn. Acad. Sci. U.S.A.* **75**, 248–252 (1978).

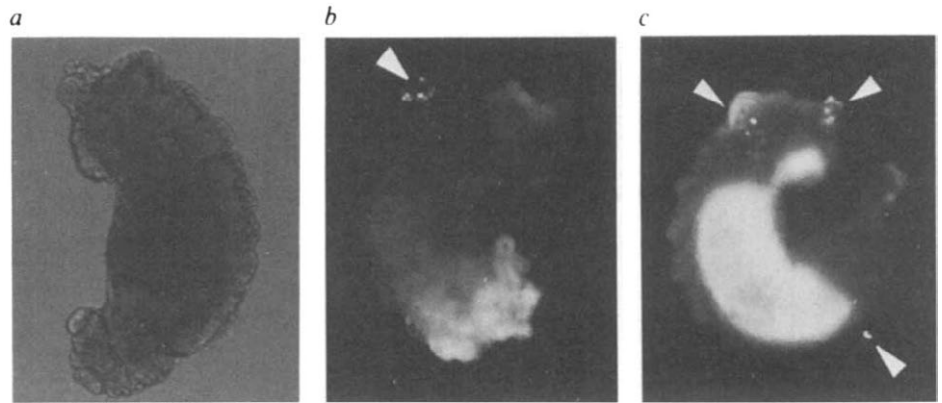
Preferential binding of imaginal disk cells to embryonic segments of *Drosophila*

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Cell recognition and selective adhesion may be important in pattern formation; such processes in *Drosophila melanogaster* could be responsible for the maintenance of segment boundaries¹, the morphogenesis of metamorphosing imaginal disks², and the paths of axon outgrowth during neurogenesis³. As cells from different imaginal disks of *Drosophila* are able to recognize and sort out from one another^{4,5}, we decided to investigate whether these larval cells could recognize and bind to the epidermis of intact embryos. We report here that imaginal disk cells bind preferentially to the epidermis of the embryonic segments from which they are derived: thoracic disk cells to thoracic segments and genital disk cells to abdominal segments. Furthermore, thoracic disk cells recognize and bind, without preference, to all segments of the homoecotic mutant *Df(3R)P9* (ref. 6). We conclude, therefore, that cells of the same segmental origin have similar recognition properties at different developmental stages.

Fig. 1 Photomicrographs of disk cells bound to the surface of *Drosophila* embryos ($\times 90$); in all three cases the anterior end of the embryo is at the top. Shown here are examples of a wild-type embryo that has been dissected out of its vitelline membrane (*a*), a wild-type embryo with thoracic disk cells attached to the second thoracic segment (*b*), and a *Df(3R)P9* embryo (*c*) with thoracic disk cells attached to several head segments (not scored) and the sixth abdominal segment. The positions of attached cells are indicated by arrowheads; the bright area in the interior of the embryos is due to autofluorescence of the yolk.



Whole imaginal disks dissected from late third-instar larvae were incubated in Schneider's⁷ insect culture medium with 5% fetal bovine serum (FBS), 2 mg ml⁻¹ collagenase and 5 μ g ml⁻¹ rhodamine-123 for 45 min, in order to disrupt the basement membranes and allow uptake of the dye. They were then rinsed in fresh medium, dissociated in 0.36 M citric acid pH 2.5 for 15 s, and immediately transferred to fresh medium containing 2.5% FBS, 1% bovine serum albumin (BSA) and Tris-base (Sigma) to neutralize the acid. With this technique, most of the disks become dissociated to single cells or small clumps⁴, with over 90% of the cells viable as determined by Trypan blue exclusion. The dissociated cells were then concentrated by settling them out at 1g onto a 100% Percoll (Sigma) cushion at room temperature.

Embryos that had just completed germ-band shortening (~ 9 h after egg deposition at 25 °C) were dissected from their vitelline membranes in Schneider's and 5% FBS using tungsten needles (Fig. 1*a*). Disk cells were then added to the embryos in a depression slide. The final volume was adjusted to 90 μ l, with

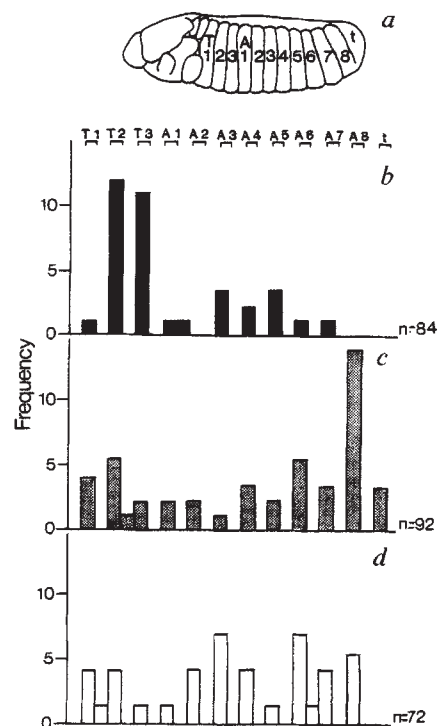
$\sim 5 \times 10^6$ disk cells ml⁻¹ for each group of 20 embryos. The mixture was incubated for 2 h at room temperature on a rocking shaker. The embryos were then rinsed twice in insect Ringer's⁸ to remove unattached cells, and scored for the position of attached fluorescent cells.

In five separate trials, mixed disk cells of thoracic origin (leg, wing and haltere disks) bound preferentially to the second and third thoracic segments of wild-type embryos (Figs 1*b*, 2*b*). From a total of 84 embryos examined, between 20 and 50% showed disk cell binding, some with multiple sites of attachment. The variation in attachment frequency between different trials was probably due to slight variations in dissociation technique, cell concentration and preparation time before incubation, because experiments done using the same disk cell preparations usually had attachment frequencies within 10% of each other.

We also examined the pattern of binding of genital disk cells to wild-type embryos, in four separate trials for a total of 92 embryos; these cells bound preferentially to the eighth abdominal segment (Fig. 2*c*), a significantly different pattern

Fig. 2 Frequencies of disk cell attachment to embryonic segments. *a*, Schematic diagram of the segmental pattern of a wild-type embryo shortly after completion of germ-band shortening, with thoracic (T), abdominal (A) and telson (t) segments labelled. *b-d*, Histograms of the frequency with which disk cells bind to a given segment for *n* embryos examined. *b*, *c*, Binding of thoracic disk cells and genital disk cells, respectively, to wild-type embryos. *d*, Binding of thoracic disk cells to *Df(3R)P9* embryos. In a few cases, disk cells attached at the boundary between segments; these were scored as intermediate between the two. For statistical comparisons, the total number of cases of binding to thoracic segments was determined for each experimental condition, as was the total number of cases of binding to abdominal segments. Using the *G* test, the totals for different experimental conditions were compared: first, thoracic disk cell as opposed to genital disk cell binding to wild-type thoracic and abdominal segments; second, thoracic disk cell binding to wild-type versus *Df(3R)P9* thoracic and abdominal segments.

Methods: Disk cells were dissociated and incubated with embryos as described in the text. Embryos were then scored for the presence of bound disk cells by fluorescence microscopy. Localized regions of the embryos were often damaged by the dissection procedure; the damage was visually apparent, with chunks of tissue either missing or mutilated. Because we found that disk cells bound indiscriminately to any regions of the embryos that had been damaged, only disk cells that were bound to apparently undamaged regions were scored. To control for the possibility that the apparently intact regions had suffered invisible damage, we varied the point at which the vitelline membrane was cut during dissection in the different trials, thus varying the regions of the embryo that were damaged. The distribution of binding to putative undamaged sites was the same for each trial, regardless of the dissection method used, indicating that the observed distribution of binding was due to cell recognition and not to undetected damage. Because the embryonic head segments were often damaged during the dissection procedures, attachment of disk cells to head segments was not considered.



($P < 0.005$ using the G test⁹) from that observed with thoracic cells. As the genital disk anlage arises from the eighth and subsequent abdominal segments¹⁰, this result indicates that disk cells are able to recognize and bind to their segments of origin.

As a further test of recognition specificity, we examined the pattern of binding of thoracic cells to embryos homozygous for a deficiency of the bithorax complex ($Df(3R)P9$). In these homozygous embryos, each segment from the third thoracic segment to the seventh abdominal segment is transformed into a mosaic of anterior mesothorax and posterior prothorax¹¹, as shown by the morphological pattern of the differentiated cuticle. Because embryos homozygous for the deficiency also fail to complete germ-band shortening when incubated at 18 °C (ref. 12), we were able to select them for use in our assay.

We performed these experiments using the same preparations of thoracic disk cells as in the experiments with wild-type embryos. In each of five trials, thoracic disk cells bound without preference to all segments of the mutant embryos (Figs 1c, 2d), a significantly different pattern ($P < 0.005$) from that observed when the same cell preparations bound to wild-type embryos. This result indicates that the homeotic transformation affects at least two aspects of segmentation: the cuticular pattern secreted by the cells of the embryonic epidermis, and the recognition properties of these cells. Also, because this result demonstrates that the mutant embryos are already transformed at the stage used, the *bithorax* genes must normally be expressed at or before this time, as concluded previously^{13,14}.

The imaginal disk anlagen arise in the embryonic epidermis at about the stage used in this assay¹⁵; it is possible, therefore, that the disk cells recognize disk anlagen rather than the epidermis as a whole. However, the disk cells do not bind only to particular regions within a segment, as would be expected if the disk cells recognized only disk anlagen. Thus, we believe that the disk cells recognize and bind to the entire epidermis of a given segment.

It should be emphasized that in these experiments the cell recognition occurs between two cell types at very different developmental stages: imaginal disk cells from late third-instar larvae (just before the onset of pupariation) and embryonic cells from a much earlier stage (~10 h after fertilization at 25 °C); thus the properties that permit their recognition and binding are present over a wide developmental period. It is possible that these recognition properties are present as early as the cellular blastoderm stage, when the cells first become committed to a particular segment identity¹⁶.

Thus, we have shown that cell recognition and selective adhesion can occur between epidermal cells of different developmental stages in *Drosophila*, and that this recognition is segment or region-specific. Such cell recognition may be a key factor in controlling developmental processes. We plan to use the assay described here to characterize the properties responsible for recognition and binding, and to determine further their specificity.

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Note added in proof: We have recently found that the preferential binding of disk cells to embryos is enhanced greatly by pretreating the embryos with 1 mg ml⁻¹ collagenase for 10 min at room temperature.

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1. Crick, F. H. C. & Lawrence, P. *Science* **189**, 340-347 (1975).
2. Poodry, C. & Schneiderman, H. *Wilhelm Roux Arch. dev. Biol.* **168**, 1-9 (1971).
3. Thomas, J. B., Bastiani, M. J., Bate, M. & Goodman, C. S. *Nature* **310**, 203-207 (1984).
4. Fehon, R. G. & Schubiger, G. *Dev. Biol.* (in the press).
5. Garcia-Bellido, A. *Dev. Biol.* **14**, 278-306 (1966).
6. Lewis, E. B. *Nature* **276**, 565-570 (1978).
7. Schneider, I. J. *exp. Zool.* **156**, 91-104 (1964).
8. Ephrussi, B. & Beadle, G. W. *Am. Nat.* **70**, 218-225 (1936).

9. Sokal, R. R. & Rohlf, F. J. *Biometry* (Freeman, San Francisco, 1969).
10. Dübendorfer, K. & Nöthiger, R. *Wilhelm Roux Arch. dev. Biol.* **191**, 42-55 (1982).
11. Hayes, P. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 545-549 (1984).
12. Gauger, A. & Schubiger, G. *Drosoph. Inf. Serv.* **60**, 108-109 (1984).
13. Morata, G. & Kerridge, S. *Nature* **290**, 778-783 (1981).
14. Struhl, G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7380-7384 (1982).
15. Madhavan, M. M. & Schneiderman, H. A. *Wilhelm Roux Arch. dev. Biol.* **183**, 269-305 (1977).
16. Simcox, A. A. & Sang, J. H. *Dev. Biol.* **97**, 212-221 (1983).

Structural identification of β - and γ -human atrial natriuretic polypeptides

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Atrial natriuretic polypeptides (ANPs) of varying chain length have been identified recently in human¹ and rat²⁻⁸ atrial tissue. Their potent natriuretic-diuretic activities indicate their key role in the regulation of extracellular fluid volume and electrolyte balance. Furthermore, human⁹ and rat¹⁰⁻¹² cDNAs encoding their precursor have been cloned and identified. Natriuretic-diuretic activity in human atrial extract comprises three distinct components (α , relative molecular mass (M_r) ~ 3,000; β , M_r ~ 6,000; γ , M_r ~ 13,000)¹. However, only the 3,000- M_r peptide, α -human atrial natriuretic polypeptide (α -hANP), comprising 28 amino acids, has so far been identified¹. We report here the purification and sequence analysis of two novel hANPs of higher M_r , β - and γ -hANP, both of which exhibit natriuretic and hypotensive activity. γ -hANP, composed of 126 amino acids, carries the α -hANP sequence at its carboxy terminus. The identification of γ -hANP reveals that the peptide, being the largest form of hANP, is processed directly from a 151-residue precursor⁹ by removal of a 26-residue signal peptide. In contrast, β -hANP (56 residues) comprises an anti-parallel dimer of α -hANP; such a dimeric peptide possessing bioactivity has never been found in the tissue as an endogenous entity.

Purification was carried out using side fractions prepared from a single human atrium, as in our previous isolation of α -hANP¹. Natriuretic activity in the extracts was monitored by an assay of relaxant activity on chick rectum¹³. At an earlier purification step, SP-Sephadex C-25 chromatography of the crude atrial was used to separate fraction SP-II, containing the less basic rectum relaxant γ -component, from the more basic fraction SP-III, which contains the α - and β -components (Fig. 1 legend). Gel filtration of fractions SP-II and SP-III yielded crude γ -hANP (13,000 M_r) and β -hANP (6,000 M_r), respectively (Figs 1a, 2a). The γ - and β -components were purified to homogeneity by subsequent cation-exchange and reverse-phase HPLC and their purity was confirmed by different HPLC systems (Figs 1b, 2b,c). The amino acid composition of the purified peptides indicates that β - and γ -hANP have 56 and 126 amino acid residues, respectively (Fig. 4 legend). From these data, the yields of β - and γ -hANP obtained from a 40-g human atrium were estimated to be 14 and 67 nmol, respectively. (Note that 92 nmol of α -hANP were isolated in the same purification.) Because γ -hANP has a disulphide linkage, sequence analysis of the peptide was performed after reductive S-carboxymethylation to convert it to the corresponding RCM (reduced and S-carboxymethylated)-derivative (Fig. 4 legend). Edman degradation combined with carboxy-terminal analysis of the RCM- γ -hANP and its peptide fragments, T1-T15 (Fig. 3a) and V1-V2, prepared by digestion with trypsin or *Staphylococcus aureus* V8 protease, unambiguously revealed the complete amino acid sequence of γ -hANP (Fig. 4), showing it to be identical with that deduced from the base sequence of cDNA encoding α -hANP⁹. Interestingly, the N-terminal sequence of γ -hANP thus determined is closely homologous to the reported N-terminal 30-residue sequence of cardiodilatin¹⁴, isolated from porcine atrial tissue as a 7,500- M_r peptide, regulating vascular smooth

muscle, although not identified fully. This suggests that cardiolatin is an N-terminal peptide of an unidentified porcine ANP.

β -hANP, having a M_r $\sim 6,000$, twice that of α -hANP, is markedly distinct from α -hANP on cation-exchange (Fig. 2b) and reverse-phase HPLC (Fig. 2c). Surprisingly, however, the amino acid composition of β -hANP was found to be identical

with that of the 3,000- M_r α -hANP (Fig. 4 legend), suggesting that β -hANP, composed of 56 residues, is a dimeric form of the 28-residue α -hANP. Amino- and carboxy-terminal analyses of β -hANP also gave identical results to those obtained with α -hANP. Furthermore, reductive *S*-carboxymethylation of β -hANP produced a single peptide of 3,000 M_r that was identical

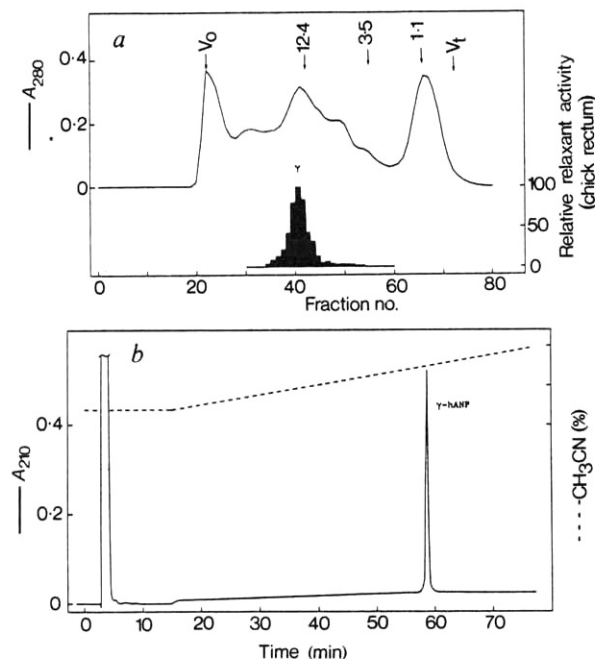


Fig. 1 *a*, Gel filtration of SP-II derived from human atrial extracts on a calibrated column of Sephadex G-75. Column, 1.8×135 cm; flow rate, 10 ml h^{-1} ; eluent, 1 M AcOH; fraction size, 5 ml per tube. Relative contractile activity on chick rectum is shown by black bars. V_0 and V_t are the initial and final volumes, respectively, and the numbered arrows indicate the M_r s ($\times 10^{-3}$) of the various peaks. *b*, Reverse-phase HPLC of purified γ -hANP (1.25 nmol). Column, TSK LS-410 ODS SIL(4×250 mm; ToyoSoda); solvent system, linear gradient elution from *a* to *b* for 80 min with a flow rate of 1 ml min^{-1} . $\text{H}_2\text{O}:\text{CH}_3\text{CN}:10\%$ trifluoroacetic acid (TFA) = *a*, 90:10:1; *b*, 40:60:1 (v/v).

Methods. The starting materials used were side fractions, referred to as the β - and γ -components of rectum activity, corresponding to about 6,000 and 13,000 M_r , respectively, which were obtained in our previous purification of α -hANP¹. Human atrial tissue (40 g) resected within 10 h post-mortem was heat-treated and extracted with 1 M AcOH containing 20 mM HCl. After desalting through an Amicon UM-2 membrane followed by acetone precipitation at a concentration of 66%, the resulting supernatant was evaporated *in vacuo* to dryness. The dry material was redissolved in 1 M AcOH and subjected to batchwise chromatography on SP-Sephadex C-25 (H^+ -form), pre-equilibrated with 1 M AcOH. Successive elutions with 1 M AcOH, 2 M pyridine and 2 M pyridine-AcOH (pH 5.0) yielded three respective fractions of SP-I, SP-II and SP-III. At this step, the γ -component was eluted in the SP-II fraction, while the α - and β -components were eluted in the SP-III fraction. The SP-II fraction thus obtained was the starting material for γ -hANP. Gel-filtration of SP-II was performed on a column of Sephadex G-75. An aliquot taken from each fraction was bioassayed for chick rectum relaxant activity (*a*). Fractions (39–44) eliciting the rectum activity were subjected to preparative HPLC on a reverse-phase column of TSK LS-410 ODS SIL(8×250 mm; ToyoSoda) and rechromatographed. The purity of the γ -hANP obtained was checked by another reverse-phase HPLC (*b*). Bioassays were carried out in the following way: (1) Chick rectum relaxant activity was assayed by methods described elsewhere^{1,13}, using strips of freshly isolated chick rectum bathed in Krebs-Henseleit solution at 37 °C. Muscle tone was induced in the rectum strips by adding carbamylcholine ($2 \times 10^{-8} \text{ M}$). (2) Natriuretic and diuretic activities were assayed as reported elsewhere⁴, after injection in rats through a jugular vein in one shot. Urine was consecutively collected every 5 min. The content of Na^+ , K^+ and Cl^- in urine samples was determined as described previously¹. Urine volumes were measured by weighing. Three observations were made for each dose.

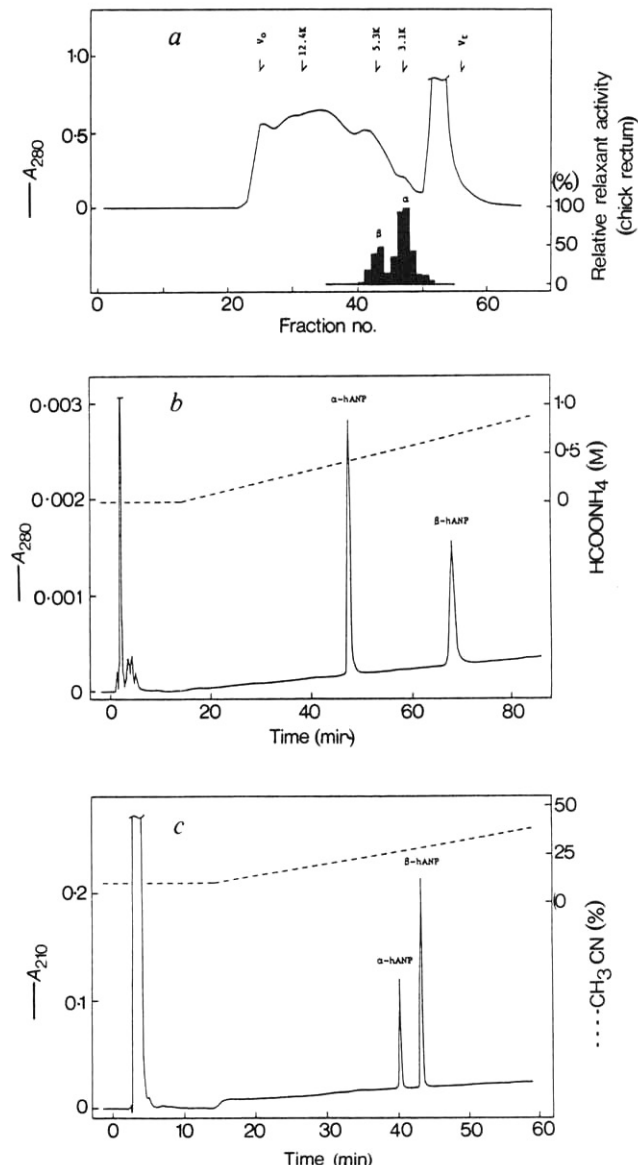


Fig. 2 *a*, Gel filtration of SP-III derived from human atrial extracts on a calibrated column of Sephadex G-50. Column, 1.2×103 cm; flow rate, 5.4 ml h^{-1} ; eluent, 1 M AcOH; fraction size, 2 ml per tube. Relative contractile activity on chick rectum is shown by black bars. *b*, Cation-exchange HPLC of purified β -hANP (0.8 nmol) compared with authentic α -hANP (1.6 nmol). Column, TSK CM-2SW (4.6×250 mm; ToyoSoda); solvent system, linear gradient elution from *a* to *b* for 80 min with a flow rate of 1 ml min^{-1} . *a*, 10 mM HCO_2NH_4 (pH 6.6): $\text{CH}_3\text{CN}=90:10$; *b*, 1 M HCO_2NH_4 (pH 6.6): $\text{CH}_3\text{CN}=90:10$ (v/v). *c*, Reverse-phase HPLC of purified β -hANP (0.5 nmol) compared with authentic α -hANP (0.5 nmol). Chromatographic conditions were as for Fig. 1b.

Methods. Purification of β -hANP from SP-III was performed similarly to that of γ -hANP (Fig. 1 legend). Gel filtration on Sephadex G-50 of SP-III yielded the β -component of rectum activity well separated from the α -component (see Fig. 2 of ref. 1). The β -component was further purified by cation-exchange HPLC (TSK CM-2SW: 7.6×300 mm; ToyoSoda), followed by reverse-phase HPLC as for the γ -component, to yield pure β -hANP. The chromatographic behaviour of purified β -hANP was compared with that of authentic α -hANP by cation exchange and reverse-phase HPLC (*c*).

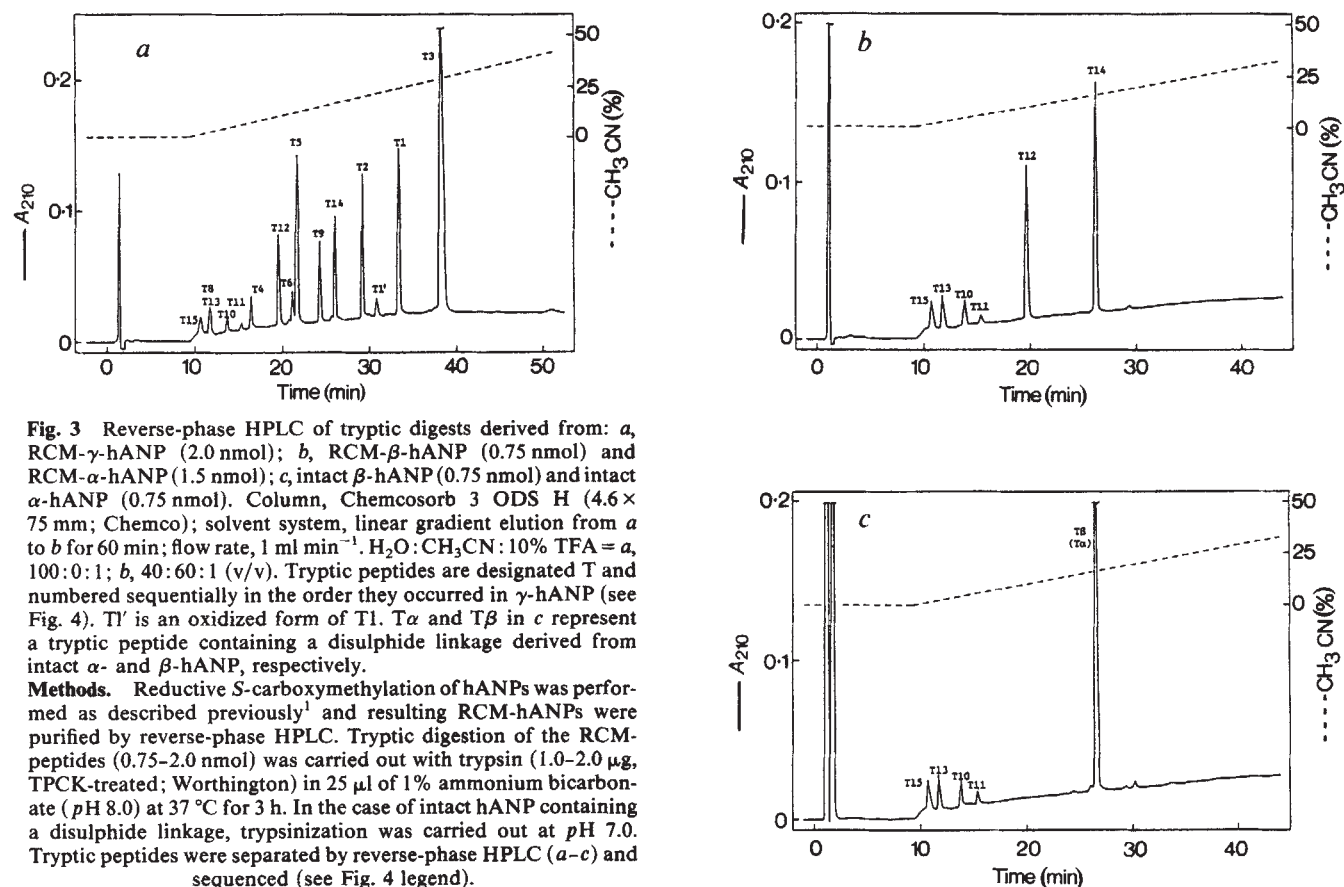


Fig. 3 Reverse-phase HPLC of tryptic digests derived from: *a*, RCM- γ -hANP (2.0 nmol); *b*, RCM- β -hANP (0.75 nmol) and RCM- α -hANP (1.5 nmol); *c*, intact β -hANP (0.75 nmol) and intact α -hANP (0.75 nmol). Column, Chemcosorb 3 ODS H (4.6 $\times$ 75 mm; Chemco); solvent system, linear gradient elution from *a* to *b* for 60 min; flow rate, 1 ml min⁻¹. H₂O:CH₃CN:10% TFA = *a*, 100:0:1; *b*, 40:60:1 (v/v). Tryptic peptides are designated T and numbered sequentially in the order they occurred in γ -hANP (see Fig. 4). T1' is an oxidized form of T1. T α and T β in *c* represent a tryptic peptide containing a disulphide linkage derived from intact α - and β -hANP, respectively.

Methods. Reductive *S*-carboxymethylation of hANPs was performed as described previously¹ and resulting RCM-hANPs were purified by reverse-phase HPLC. Tryptic digestion of the RCM-peptides (0.75–2.0 nmol) was carried out with trypsin (1.0–2.0 μ g, TPKC-treated; Worthington) in 25 μ l of 1% ammonium bicarbonate (pH 8.0) at 37 °C for 3 h. In the case of intact hANP containing a disulphide linkage, trypsinization was carried out at pH 7.0. Tryptic peptides were separated by reverse-phase HPLC (*a*–*c*) and sequenced (see Fig. 4 legend).

with the authentic RCM- α -hANP (Fig. 4 legend). A tryptic peptide map prepared from RCM- β -hANP was also identical with that from RCM- α -hANP (Fig. 3*b*). All these results indicate that β -hANP is a dimeric form of α -hANP. Furthermore, the tryptic peptide map derived from the intact β -hANP is identical with that from the intact α -hANP, indicating that β -hANP is probably an antiparallel dimer of α -hANP. Trypsinization of the intact α - and β -hANP at pH 7.0 produced an identical peptide (T α and T β) containing a disulphide linkage, along with four other identical peptides (T10, T11, T13 and T15) (Fig. 3*c*). Both T β and T α produced T12 and T14 by reductive *S*-carboxymethylation, indicating that T β is a peptide in which Cys 7 in T12 is linked intermolecularly with Cys 23 in T14. Accordingly, we conclude that β -hANP is an antiparallel dimeric form of α -hANP linked by two sets of intermolecular crosslinks between Cys 7 and Cys 23. The possibility that β -hANP is a parallel dimer is excluded because there are no tryptic fragments involving Cys 7–Cys 7 or Cys 23–Cys 23 crosslinking in the tryptic digests of β -hANP. Thus, the complete structure of β -hANP has been determined (Fig. 4). Although breakage and rejoining of a disulphide linkage in peptides is conceivable, particularly at high pH, it was confirmed that the synthetic α -hANP, despite its exposure to the conditions used for the

present purification, did not undergo dimerization to β -hANP. In addition, β -hANP of 6,000 *M_r* was always observed in the initial extract of other human atrial tissues, indicating that β -hANP is present in the tissue as an endogenous entity, even though the way in which such an unusual dimerization takes place *in vivo* remains unknown.

Intravenous injection of purified β - and γ -hANP in rats resulted in marked increases in the excretion of urine and electrolytes. The responses induced by γ -hANP were maximal within 5 min of injection and essentially complete within 30 min, in a manner similar to α -hANP¹. However, the natriuretic-diuretic response of β -hANP showed a slower onset and longer duration of action than α - and γ -hANP. A response was first observed 5 min after injection; β -hANP-induced natriuresis and diuresis were maximal in the subsequent 5 min and lasted for 50–60 min after injection. Because of the very small amounts available, biological activities of β - and γ -hANP have not been compared accurately. However, preliminary data (Table 1) may give an approximate estimate of their biological activity.

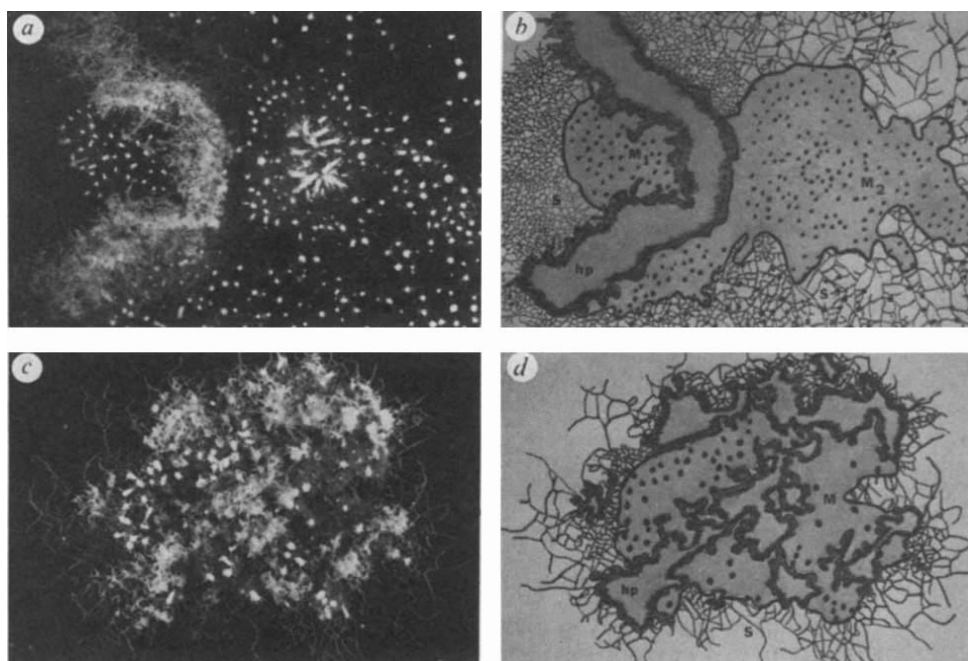
Based on these data, natriuretic-diuretic responses induced by β -hANP (0.8 nmol) and γ -hANP (1.0 nmol) seem to be comparable to those induced by α -hANP (0.2 nmol). However, injection of 1.0 nmol α -, β - and γ -hANP in anaesthetized rats caused a comparable decrease in blood pressure (maximum 15–20 mmHg), lasting 50–60 min. Thus, the hypotensive responses to α -, β - and γ -hANP are not as great as their natriuretic activities, perhaps implying a mechanistic dissociation of natriuretic and hypotensive activities. It has been reported that reductive *S*-carboxymethylation of α -hANP abolishes its natriuretic activity, suggesting that a disulphide linkage or ring-structure of α -hANP is essential for activity^{1,3}. Interestingly, however, β -hANP lacks the ring-structure observed in α -hANP, and yet exerts appreciable natriuretic activity. Because our β -hANP preparation was chromatographically pure and totally free from α -hANP contamination, it is evident that β -hANP shows natriuretic and hypotensive activities of its own. Thus, β -hANP may retain a structural conformation resembling the ring-structure of α -hANP which is required for activity. The

Table 1 Diuretic and natriuretic responses induced by α -, β - and γ -hANP

Dose injected (nmol)	α -hANP 0.2	β -hANP 0.8	γ -hANP 1.0
Urine output	315 $\pm$ 52	298 $\pm$ 42	212 $\pm$ 38
Na ⁺ excretion	422 $\pm$ 83	335 $\pm$ 78	295 $\pm$ 52
K ⁺ excretion	278 $\pm$ 48	152 $\pm$ 27	143 $\pm$ 12
Cl ⁻ excretion	451 $\pm$ 78	366 $\pm$ 62	320 $\pm$ 49

Diuretic and natriuretic responses expressed as % change (mean $\pm$ s.e.) in urine output and in excretions of Na⁺, K⁺ and Cl⁻ from 15-min urine samples collected before and after intravenous injection in anaesthetized rats. Three rats were used for each sample.

Fig. 1 Historecognition in *Hydractinia echinata*. *a*, Rejection between two incompatible colonies. Note the broad zone of hyperplastic tissue separating the two colonies. *b*, Diagram of *a*: hp, hyperplastic stolons; $M_{1,2}$, ectodermal mat of colonies 1 and 2; S, stolons; dots represents polyps. *c*, An autoreactive colony. Note the development of hyperplastic stolons throughout the colony and that this response has been initiated in the absence of contact with other *H. echinata* colonies. Contrast this morphology with the right-hand side of *a*, which shows the morphology of a normal colony. *d*, Diagram of *c*. Colonies were bred and maintained in the laboratory using methods described in detail elsewhere^{8,9}.



(Fig. 1*a, b*). Within 24 h of contact, the stolons begin to swell, due to a large-scale migration of multipotent interstitial cells (I-cells) from the central mat. These hyperplastic stolons lift from their substratum and their epithelial surface is replaced by a layer of specialized cells. These cells contain basotrichous isorhizal nematocysts⁹, harpoon-like structures which discharge into the foreign tissue and effect its destruction. The discharged nematocysts are sloughed off, a new set differentiates from I-cells, and the histocompatibility reaction continues until one colony has eliminated the other⁹.

A large number of offspring from crosses between three normal male and three normal female lines were tested for histocompatibility responses, and a small percentage of autoreactive offspring developed from one of the females (Table 1). These colonies were raised from single zygotes and had no contact with other *H. echinata* colonies, hence chimaerism cannot explain their autoreactivity. Stolons of autoreactive colonies

often failed to fuse, in contrast to normal colonies. Most strikingly, however, autoreactive colonies, on reaching a characteristic size, spontaneously developed a morphology resembling rejection of incompatible colonies, but did so in the absence of any other individual (Fig. 1*c, d*). The onset of the autoreactive episode occurred at a size at which germ cells matured in normal siblings (mean size at onset of reproduction in siblings: 240 polyps, $n=6$; mean size at onset of autoreactivity: 313 polyps, $n=9$; $P>0.3$, t -test). However, autoreactive colonies failed to reach reproductive maturity. Regions of hyperplasticity developed in areas of stolonal growth, then grew towards the central region of the colony, effecting the destruction of their own tissues. Destruction of autologous tissues was not lethal, however. The hyperplastic response is initiated only by stolons⁹, and once these stolons were destroyed the colony reinitiated growth. This 'remission' was temporary, autoreactivity being triggered again at a characteristic size. Thus, colony size cycled, with growth during periods of remission and shrinkage during autoreactive episodes (Fig. 2).

Ultrastructural observations of the autoreactive condition revealed batteries of nematocysts in the hyperplastic stolons indistinguishable from those of normal hyperplastic stolons. Autoreactive colonies, despite their defects in self-tolerance, retained typical histocompatibility relationships with allogeneic tissue; they fused with parents, siblings and half-siblings, but mounted a hyperplastic response to unrelated colonies (Table 2), as is typical of this lineage. Thus, autoreactive colonies display a maternally inherited defect (Table 1) which has no apparent effect on normal tissue rejection mechanisms, but periodically directs these same mechanisms towards the destruction of their own tissues.

Colonial organisms, unlike vertebrates, do not sequester their germ lines at an early stage of ontogeny, but rather differentiate

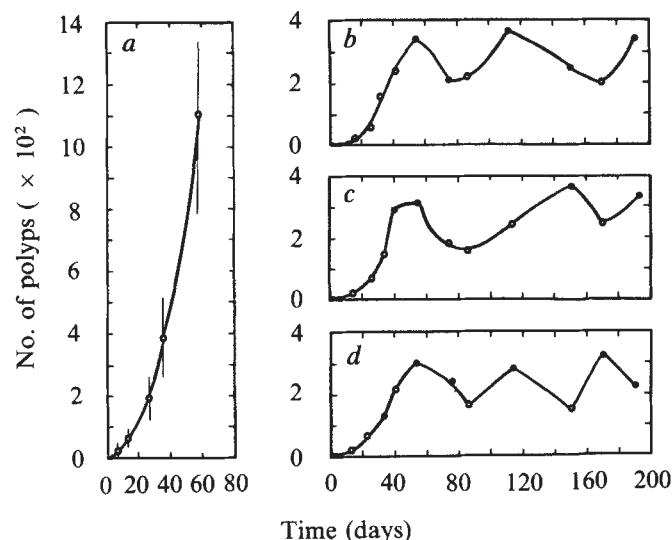


Fig. 2 Growth of normal and autoreactive colonies. *a*, Number of polyps as a function of time, expressed as the mean and 1 s.d., for two parents and four normal siblings of autoreactive colonies. *b-d*, Growth of three autoreactive colonies. Solid points represent hyperplastic episodes; open points represent periods of remission. Note that size in these colonies cycles with time.

Table 1 Frequency of autoreactivity

		Females		
		A	B	C
Males	D	5 (55)	0 (32)	0 (45)
	E	3 (51)	0 (129)	0 (29)
	F	7 (94)	0 (153)	0 (61)

Values given are the number of autoreactive colonies; the numbers in parentheses are the sample sizes.

Table 2 Histocompatibility responses of normal and autoreactive individuals

Test strain	Autoreactive colonies			Normal siblings		
	<i>n</i>	Fusion	Rejection	<i>n</i>	Fusion	Rejection
Parents	8	8	0	104	101	3
Normal sibs	48	48	0	111	108	3
Autoreactive sibs	6	6	0	36	108	3
Half-sibs	36	35	1	126	117	9
Unrelated	21	0	21	43	0	43

germ cells from a pool of multipotent stem cells¹⁰. In *Hydractinia*, the capacity for allorecognition appears well before reproductive maturation⁶⁻⁹. The autoreactive condition, however, arises coincidentally with the acquisition of reproductive maturity. Thus, autoreactivity may represent a response to self-markers expressed only with the onset of germ-cell maturation. The observation that the autoreactive condition fails to disrupt normal allorecognition (Table 2) further supports the suggestion that autoreactivity is a response to self-markers other than those associated with allorecognition. It is tempting to draw the analogy (or homology?) between this invertebrate defect in self-tolerance with the well-known association in vertebrates of reproductive maturation and the onset of various autoimmune defects¹¹.

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1. Burnet, F. M. *Self and Not Self* (Cambridge University Press, 1970).
2. Burnet, F. M. *Nature* **232**, 230-235 (1971).
3. Hildemann, W. H. *Nature* **293**, 116-120 (1977).
4. Marchalonis, J. J. *Immunity in Evolution* (Harvard University Press, Cambridge, 1977).
5. Buss, L. W. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5337-5341 (1982).
6. Hauenschild, W. *Wilhelm Roux Arch. Entw. Mech.* **147**, 1-114 (1954).
7. Hauenschild, W. *Z. Naturf.* **11**, 132-174 (1956).
8. Ivker, F. *Biol. Bull.* **143**, 162-174 (1972).
9. Buss, L. W., McFadden, C. S. & Keene, D. R. *Biol. Bull.* **167**, 131-158 (1984).
10. Buss, L. W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1387-1391 (1983).
11. Smith, H. R. & Steinberg, A. D. *Rev. Immun.* **1**, 175-210 (1983).

Unique structure of murine interleukin-2 as deduced from cloned cDNAs

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Interleukin-2 (IL-2) is a lymphokine originally described as a humoral factor required for the continued proliferation of activated T-cell clones^{1,2}. It also seems to be involved in the mitogenic response of thymocytes^{3,4}, in augmenting natural killer cell activity⁵, in the generation of cytotoxic T cells⁶ and in the induction of other lymphokines such as γ -interferon^{7,8} and a B-cell growth factor (BCGF-1)⁹. More recently, there has been evidence for the involvement of IL-2 *per se* in the stimulation of B-cell growth (ref. 10 and T. Kishimoto and J. Vilček, personal communications). We have reported previously the cloning and expression of a human IL-2 complementary DNA. The cDNA encodes biologically active IL-2 which would consist of 153 amino acids, including a signal sequence¹¹. Because so much of the work on IL-2 has been done in the human and mouse, we sought to obtain cDNA encoding murine IL-2, and we now report the cloning, expression and sequence analysis of murine IL-2 cDNAs. The longest cDNA insert encodes a polypeptide of 169 amino acids, containing unique repeats of a CAG sequence which would encode 12 consecutive glutamine residues within the active IL-2 molecule.

Polyadenylated RNA was isolated from a mouse lymphoma cell line, LBRM-33 (ref. 12), 6 h after stimulation by phytohaemagglutinin (PHA, final concentration 1%) and fractionated by centrifugation on a sucrose gradient¹¹ by the published procedure. Fractions containing messenger RNA whose

sequence cross-hybridized to the cloned human IL-2 cDNA were monitored by blotting analysis of the single-stranded cDNA synthesized on aliquots of each mRNA fraction. Specific hybridization was observed in non-stringent conditions (see Fig. 1a legend) in a mRNA fraction corresponding to 12S (results not shown), and RNA from this fraction was used to prepare a cDNA library by the standard procedure^{11,13}. Clones which cross-hybridized to the human IL-2 cDNA sequence (the largest *Hinf*I fragment)¹¹ were screened by the *in situ* procedure¹⁴, and from 15,000 colonies screened, we identified three positive clones (numbers 14, 20 and 21). The cDNA insert from each plasmid, designated pMIL2-14, pMIL2-20 and pMIL2-21, respectively, shared common sequences with each other, but none covered the entire 5' region of the mRNA (Fig. 1a). We therefore prepared another cDNA library and screened it (~10,000 colonies) using the nick-translated cDNA insert from pMIL2-14 as probe. Two positive clones were identified and each of their plasmids, pMIL2-45 and pMIL2-58, contained a cDNA insert consisting of ~1,000 base pairs (bp) (Fig. 1a).

The complete nucleotide sequence of the cDNA insert from pMIL2-45 was determined and compared with the human IL-2 cDNA (Fig. 1b). The cDNA insert of pMIL2-45 consists of 940 bp, with a stretch of A residues corresponding to the poly(A) tail of the mRNA. The cDNA sequence contains a large open reading frame which encodes 169 amino acids. Like the human IL-2, 20 amino acids at the N-terminal region would constitute a signal peptide which is cleaved off during secretion. A 76%

Table 1 Detection of IL-2 activity in the culture media of COS cells transfected by pCM-45

Transfected DNA	IL-2 activity (U ml ⁻¹)
pCM-45	835
pCMΔ-45	<0.1
pCE-1	<0.1
None	<0.1

Intact pCM-45, pCMΔ-45 and pCE-1 were transfected into monkey COS cells¹⁵ essentially as described previously¹¹, and 72 h after transfection, IL-2 activity was assayed by the procedure described by Gillis *et al.*².

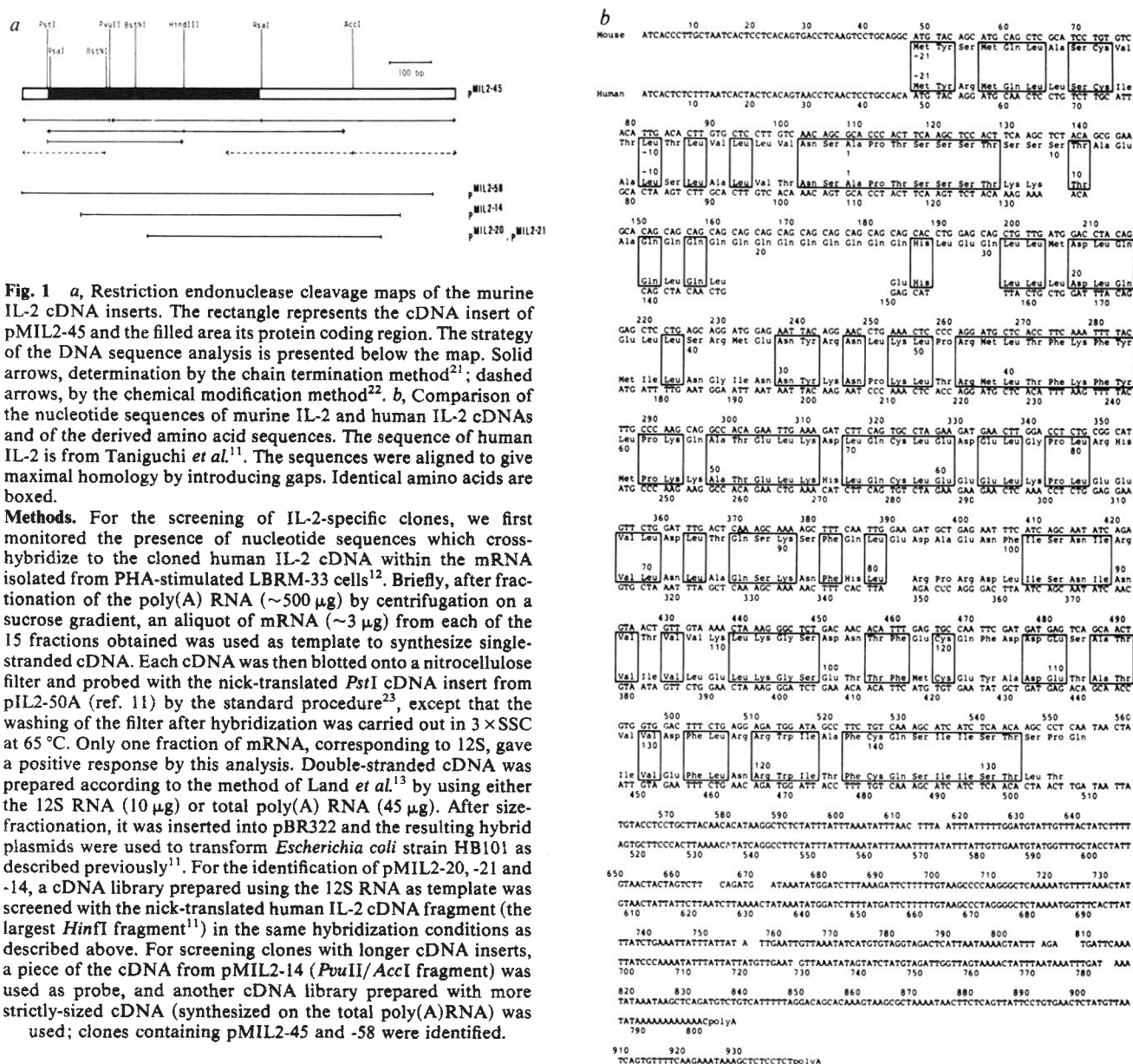


Fig. 1 *a*, Restriction endonuclease cleavage maps of the murine IL-2 cDNA inserts. The rectangle represents the cDNA insert of pMIL-2-45 and the filled area its protein coding region. The strategy of the DNA sequence analysis is presented below the map. Solid arrows, determination by the chain termination method²¹; dashed arrows, by the chemical modification method²². *b*, Comparison of the nucleotide sequences of murine IL-2 and human IL-2 cDNAs and of the derived amino acid sequences. The sequence of human IL-2 is from Taniguchi *et al.*¹¹. The sequences were aligned to give maximal homology by introducing gaps. Identical amino acids are boxed.

Methods. For the screening of IL-2-specific clones, we first monitored the presence of nucleotide sequences which cross-hybridize to the cloned human IL-2 cDNA within the mRNA isolated from PHA-stimulated LBRM-33 cells¹². Briefly, after fractionation of the poly(A) RNA (~500 µg) by centrifugation on a sucrose gradient, an aliquot of mRNA (~3 µg) from each of the 15 fractions obtained was used as template to synthesize single-stranded cDNA. Each cDNA was then blotted onto a nitrocellulose filter and probed with the nick-translated *Pst*I cDNA insert from pMIL-2-50A (ref. 11) by the standard procedure²³, except that the washing of the filter after hybridization was carried out in 3 × SSC at 65 °C. Only one fraction of mRNA, corresponding to 12S, gave a positive response by this analysis. Double-stranded cDNA was prepared according to the method of Land *et al.*¹³ by using either the 12S RNA (10 µg) or total poly(A) RNA (45 µg). After size-fractionation, it was inserted into pBR322 and the resulting hybrid plasmids were used to transform *Escherichia coli* strain HB101 as described previously¹¹. For the identification of pMIL-2-20, -21 and -14, a cDNA library prepared using the 12S RNA as template was screened with the nick-translated human IL-2 cDNA fragment (the largest *Hinf*I fragment¹¹) in the same hybridization conditions as described above. For screening clones with longer cDNA inserts, a piece of the cDNA from pMIL-2-14 (*Pvu*II/*Acc*I fragment) was used as probe, and another cDNA library prepared with more strictly-sized cDNA (synthesized on the total poly(A)RNA) was used; clones containing pMIL-2-45 and -58 were identified.

nucleotide sequence homology was found between the murine and human IL-2 cDNA, whereas comparison of the deduced amino acid sequence with that of human IL-2 revealed 63% homology at the protein level, including the signal peptide, without taking into account the regions where gaps were introduced to maximize the overall homology.

The most peculiar feature of the murine IL-2 cDNA sequence is the tandem repeats of a CAG sequence in the protein coding region (nucleotides 150–185) which would produce 12 consecutive glutamine residues in the IL-2 polypeptide. The repeat occurs in other IL-2-specific cDNAs cloned independently (see Fig. 1a) as well as in the first exon of the chromosomal gene isolated from another mouse cell line (A. Fuse and T.T., unpublished results). Hence, it is most likely to be a genuine sequence which cannot be attributed to a cloning artefact or to a peculiarity of the cell line used here.

To confirm that the cloned cDNA encodes biologically active IL-2, the cDNA insert was excised by *Pst*I cleavage of pMIL-2-45 and linked to a vector, pCE-1 (ref. 11), to demonstrate IL-2 expression in monkey COS cells (pCM-45)¹⁵. We also modified the cDNA, so that the ATG sequence at nucleotides 204–206 would function as the initiator in the mRNA, by cleaving the cDNA insert with *Pvu*II, as this single cleavage site (nucleotides

195–200) is located just upstream of the ATG and downstream of the CAG repeat (pCMΔ-45) (Fig. 2). COS cells transfected with pCM-45 but not with pCMΔ-45 secreted active IL-2 molecules into the culture media (Table 1), and the IL-2 produced showed many properties seen in authentic murine IL-2 (results not shown). Taken together, these results demonstrate that the stretch of 12 consecutive glutamines in fact constitutes a part of the murine IL-2 molecule. The CAG repeats could have arisen either by direct insertion of the whole sequence or by amplification of a unit sequence. It is interesting that a sequence, CAG-CTA-CAA-CTG-GAG-CAT (nucleotides 138–155), is present in the corresponding region of the human IL-2 gene. Thus, the CAG repeat in the mouse IL-2 gene may have been generated by duplication of a primordial sequence resembling the human sequence. Another repeated sequence (nucleotides 114–137) occurs in the upstream region of the CAG repeats and the related sequence is present only once in the human counterpart (nucleotides 114–125).

As deduced from the cDNA sequence, the mature murine IL-2 would contain 149 amino acids and its relative molecular mass (M_r) was calculated to be 17,233.5, compared with an estimated M_r for the native murine IL-2 of 15,500–31,000^{16–18}. As for human IL-2, there is no potential *N*-glycosylation site

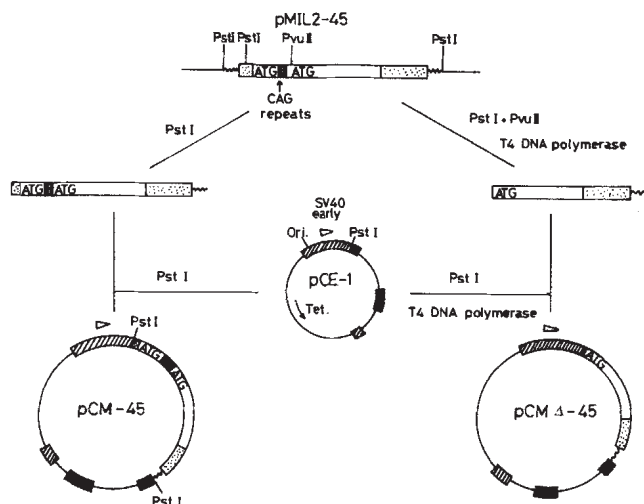


Fig. 2 Construction of plasmid DNAs for the expression of murine IL-2 cDNA. Plasmid pCE-1, which contains the simian virus 40 (SV40) early promoter¹¹, was cleaved at the single *Pst*I site and ligated to the IL-2 cDNA which was excised by *Pst*I digestion of pMIL2-45 to construct pCM-45. To construct pCMΔ-45, the cDNA was excised by *Pvu*II and *Pst*I. The *Pst*I cleavage site was rendered flush by T4 DNA polymerase and then ligated to *Pst*I-cleaved pCE-1 DNA whose ends were also made flush by T4 DNA polymerase treatment. In those plasmids, expression of the IL-2 structural gene should be under the control of the viral promoter. Ori, origin; ▨, noncoding region; ▨, CAG repeats; □, coding region; ▨, SV40 sequence; ▨, rabbit β-globin gene; and ~, G-C tails.

in the murine IL-2 sequence. Three cysteine residues, two of which (positions 58 and 105) seem to have a critical role in maintaining the human IL-2 molecule in the active conformation¹⁹, are conserved also in the mouse IL-2 sequence (positions 72, 120 and 140). At present, we have no evidence for other genes encoding active IL-2 in the mouse genome. Thus, the molecular heterogeneity of the native murine IL-2¹⁸ may be due to the different extent of post-translational modifications of a single product, as reported for human γ-interferon²⁰. The murine IL-2 produced by a cloned gene will be useful for the study of the biological properties of this lymphokine as well as for the evaluation in mice of the clinical potential of IL-2.

We thank Dr Y. Gulzman for COS cells, Drs T. Miyata and A. Fuse for valuable comments and Dr H. Sugano for support. **Note added in proof:** We have now learned that two groups have obtained mouse IL-2 clones containing essentially the same sequence as ours (K. Arai and W. Fiers, personal communications).

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- Morgan, D. A., Ruscetti, F. W. & Gallo, R. *Science* **193**, 1007-1008 (1976).
- Gillis, S., Ferm, M. M., Ou, W. & Smith, K. *J. Immunol.* **120**, 2023-2027 (1978).
- Chen, B. M. & Di Sabato, G. *Cell. Immunol.* **22**, 211-224 (1976).
- Shaw, J., Monticone, V. & Paetkau, V. *J. Immunology* **120**, 1967-1973 (1978).
- Henney, C. S., Kuribayashi, K., Kern, D. E. & Gillis, S. *Nature* **291**, 335-338 (1981).
- Wagner, H., Hardt, C., Heeg, K., Rollinghoff, M. & Pfizenmaier, K. *Nature* **284**, 278-280 (1980).
- Farrar, W. L., Johnson, H. M. & Farrar, J. J. *J. Immunol.* **126**, 1120-1125 (1981).
- Pearlstein, K., Palladino, M. A., Welte, K. & Vilček, J. *Cell. Immunol.* **80**, 1-9 (1983).
- Howard, M. *et al. J. exp. Med.* **158**, 2024-2039 (1983).
- Korsmeyer, S. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 4522-4526 (1983).
- Taniguchi, T. *et al. Nature* **302**, 305-310 (1983).
- Gillis, S., Scheild, M. & Watson, J. D. *J. Immunol.* **125**, 2570-2580 (1980).
- Land, H., Grez, N., Hansen, H., Lindermaier, W. & Schuetz, G. *Nucleic Acids Res.* **9**, 2251-2266 (1981).
- Grinstein, M. & Hogness, D. S. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2230-2234 (1975).
- Gulzman, Y. *Cell* **23**, 175-182 (1981).
- Granelli-Piperno, A., Vassalli, J.-D. & Reich, E. *J. exp. Med.* **154**, 422-431 (1981).
- Caplan, B., Gibbs, C. & Paetkau, V. *J. Immunol.* **126**, 1351-1354 (1981).
- Gillis, S. *et al. Immun. Rev.* **63**, 167-209 (1982).
- Wang, A., Lu, S. D. & Mark, D. F. *Science* **224**, 1431-1433 (1984).
- Rinderknecht, E., O'Connor, B. H. & Rodoriguez, H. *J. biol. Chem.* **259**, 6790-6797 (1984).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Maxam, A. & Gilbert, W. *Methods Enzymol.* **65**, 560-580 (1980).
- Ohno, S. & Taniguchi, T. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5305-5309 (1980).

Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma

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Proto-oncogenes may be important in the cellular processes central for the growth and differentiation of normal cells^{1,2}. N-myc is a DNA sequence which shares limited homology to the proto-oncogene c-myc and has been found to be amplified in both primary tissue³ and cell lines from neuroblastoma^{4,26}, a childhood tumour of neuroectodermal origin. Differentiation of this embryonal tumour is of clinical importance, since occasional tumours have been noted to differentiate *in vivo* to benign ganglioneuroma^{5,27,28}. *In vitro*, many human neuroblastoma cell lines can be induced to differentiate morphologically and biochemically by a variety of agents⁶⁻¹¹. Retinoic acid (RA), an analogue of vitamin A, has been shown to inhibit neuroblastoma cell growth and clonability in soft agar, and to induce extensive neurite outgrowth^{8,9}. Therefore we examined the relationship of N-myc expression to the *in vitro* differentiation of these cells. We report here that in the case of RA-induced differentiation, a decreased level of expression is detected within 6 h of treatment and precedes both cell-cycle changes and morphological differentiation.

To study the relationship between N-myc expression and neuroblastoma differentiation, log-phase SMS-KCNR neuroblastoma cells¹² were incubated in medium containing RA or the solvent control, ethanol. At various times after the initiation

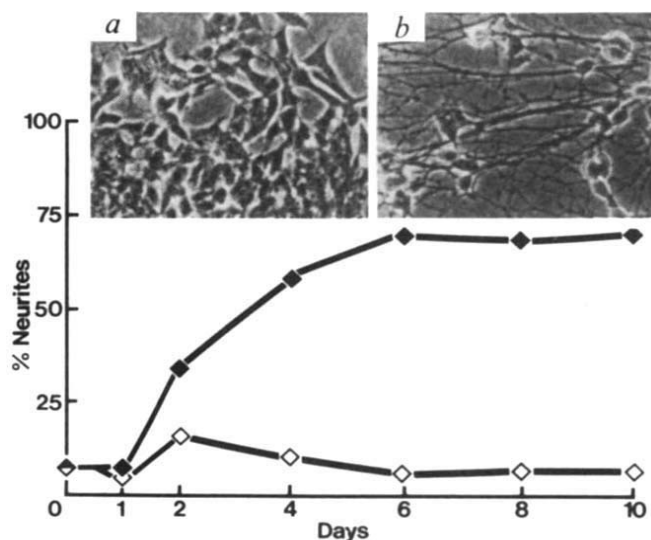
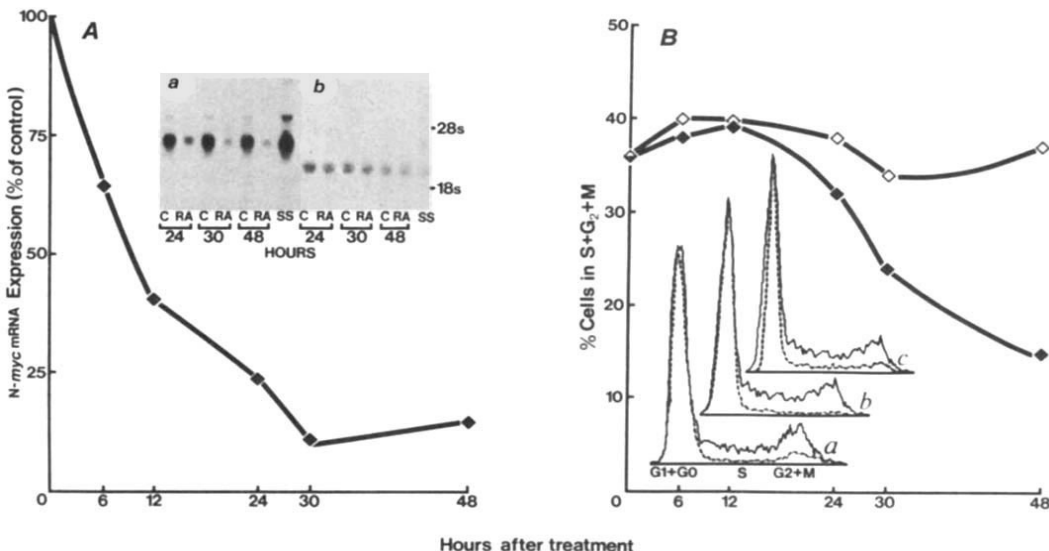


Fig. 1 Effect of RA on neurite outgrowth. ♦, RA treated; ◇, ethanol treated. The inset photographs show SMS-KCNR cells after 10 days of treatment with ethanol (a) and with retinoic acid (b). SMS-KCNR cells¹² were plated in 150-mm tissue culture dishes in RPMI 1640 with 15% fetal calf serum and incubated at 37 °C with 5% CO₂. After 2 days of incubation, the medium was removed and replaced with medium containing 1 × 10⁻⁵ M trans-retinoic acid (Sigma) dissolved in ethanol at a concentration of 5 mM or control medium containing ethanol. For determination of neurite outgrowth, the number of cells extending a neurite longer than the cell body were counted and expressed as a percentage of the total number of cells counted. Each data point represents the average of five random fields from each plate of triplicate cultures.

Fig. 2 A, Graphic representation (from densitometer scans of autoradiograms) of the relative levels of N-myc expression in RA-treated SMS-KCNR cells compared with control cells treated with ethanol for various periods. Serum-starved cells are also examined. Inset *a*, shows a Northern blot of cytoplasmic RNA from treated (RA) and control (C) cells, hybridized with 32 P-labelled N-myc DNA, and *b* shows the same Northern blot hybridized with 32 P-labelled c-myc DNA. B, A graphic presentation of the percentage of cells in the growth fraction or S+G₂+M in the ethanol-treated control cultures (◇) compared with those treated with 10×10^{-6} M trans-retinoic acid (◆). The inset DNA histograms show cell-cycle profiles for SMS-KCNR comparing: *a*, control cultures (—) and RA treatment at 48 h (---); *b*, control cultures (—) and isoleucine-depleted cultures (---); *c*, control cultures (—) and serum-deprived cultures (---).



Methods. A, 5×10^6 SMS-KCNR cells were plated, cultured and treated as described in Fig. 1 legend. At various times after treatment, duplicate experimental and control plates were collected for cell-cycle analysis and isolation of cytoplasmic RNA. RNA was isolated by lysing the cells with 0.5% Nonidet P-40, spinning out the nuclei, extracting the supernatant with phenol followed by chloroform-isoamyl alcohol extraction and ethanol precipitation. Samples of 20 μ g of total cytoplasmic RNA from the various time points indicated were denatured in 2.2 M formaldehyde, 50% formamide, 0.2 M MOPS, 50 mM sodium acetate, 1 mM EDTA, pH 7.0 for 5 min at 65 °C. The samples were electrophoresed in a 1% agarose-formaldehyde/MOPS-buffered gel. The RNA species were transferred to nitrocellulose and hybridized with nick-translated pNb-1 DNA specific for N-myc⁴, in conditions of high stringency (42 °C, 50% formamide, 10% dextran sulphate) washed at 60 °C in 0.2 $\times$ SSC, 0.1% SDS and exposed to X-Omat AR film for 16 h with a Lightening-plus intensifying screen at -70 °C. For detection of c-myc expression, the same nitrocellulose blot was rehybridized with nick-translated p5PP DNA containing c-myc¹⁶, washed at 55 °C in 0.2 $\times$ SSC, 0.1% SDS and the autoradiogram was exposed for 7 days as described above. B, For cell-cycle analysis, cells were plated and treated with RA or ethanol as described in A. At the appropriate time, cells were collected as previously described⁶ to form a cell suspension. Viability ($\geq 85\%$) was determined by haemocytometer using 0.07% Trypan blue and 1×10^6 cells were stained in 0.05 mg ml⁻¹ propidium iodide in 0.1% citrate²³ + 0.05 mg ml⁻¹ RNase on ice and analysed using an Ortho cytofluorograph as previously described²⁴. RA-treated and control cells collected for cell-cycle analysis showed no difference in viability during the first 48 h of culture, excluding the possibility that the N-myc decrease and cell-cycle arrest were due to selective cell death (data not shown). A total of 8×10^3 cells were analysed for each data point, and the percentage of cells in G₁, S and G₂+M was determined by modification of a graphical curve fitting method²⁵, calibrated using the polynomial curve fitting software resident on the Ortho 2150 computer²⁴.

of treatment, the cells were examined for morphological differentiation as assessed by neurite outgrowth and collected for cell-cycle analysis and isolation of cytoplasmic RNA. Neurite outgrowth is the hallmark of neuroblastoma *in vitro* morphological differentiation⁶⁻⁹. The time course of neurite extension for SMS-KCNR cells shows that maximal morphological differentiation (69.5% of the cells extending measurable neurites) was reached 6 days after the start of RA treatment (Fig. 1). There was no detectable difference in neurite outgrowth of RA-treated cells compared with controls at 6 h and only a small portion of the cells had extended neurites by 24 h (8.7% in RA treated cells; 3.9% in the control).

N-myc expression was assessed by hybridization of DNA from plasmid pNb-1 to Northern blots of cytoplasmic RNA from RA or control cultures (Fig. 2A). A detectable decrease in the levels of RNA hybridizing to N-myc was observed in RA-treated SMS-KCNR cells by 6 h after treatment (Fig. 2A, inset *a*). By 30–48 h after treatment, there was an 85% decrease in the level of N-myc expression in RA-treated cultures compared with control cultures and expression remained at approximately this level during the 10-days of our experiment (Fig. 3). The decreased N-myc in neuroblastoma cells treated with RA is not due to selective loss of amplified N-myc sequences, since Southern blot analysis of SMS-KCNR neuroblastoma cells treated with RA for 14 days showed no difference in N-myc copy number compared with untreated controls (data not shown).

Cell-cycle analyses of parallel cultures of SMS-KCNR revealed that the percentage of cells in the growth fraction (S+G₂+M) was not significantly different until 24 h after the addition of RA (Fig. 2B). At 48 h, when N-myc expression was maximally depressed, cell-cycle arrest was also maximal, with

14% of the RA-treated cells in the growth fraction compared with 37% in the control culture (Fig. 2B, inset *a*). To determine if the decrease in N-myc expression could be due to growth arrest, we blocked SMS-KCNR cells in the G₁ phase by either serum starvation or isoleucine deprivation. Neither of these treatments induced neurite outgrowth of SMS-KCNR. Incubation of cells in serum-free or isoleucine-depleted medium for 4 days resulted in 18.5% and 12% of the cells in the growth fraction, respectively (Fig. 2B, insets *b*, *c*). Northern analysis of cytoplasmic RNA revealed that there was no decrease in N-myc expression in serum-starved cells (Fig. 2A, inset *a*) or isoleucine-depleted cells (data not shown). These results indicated that the decrease in N-myc expression preceded a detectable change in cell cycle and that arresting the cells in G₁ was not sufficient to down-regulate N-myc levels.

RA-induced differentiation of the human myeloid leukaemia cell line HL60 to granulocytes¹³ and the F9 murine teratocarcinoma cell line¹⁴ to parietal endoderm is accompanied by decreased levels of expression of the proto-oncogene c-myc^{2,15}. Since N-myc shares limited homology with c-myc, we examined the expression of c-myc in RA-treated SMS-KCNR cells. The Northern blot previously used for detection of N-myc was rehybridized with c-myc containing plasmid DNA¹⁶ (Fig. 2A). The results of this experiment indicate no significant difference between the steady-state levels of c-myc in RA-treated and control cultures at times when N-myc expression was dramatically altered by RA (Fig. 2A, inset *b*). Thus, the proto-oncogene c-myc and N-myc appear to be differentially regulated in RA-treated neuroblastoma cells.

We also studied the steady-state levels of cytoplasmic N-myc expression in several other neuroblastoma cell lines treated with a variety of agents known to induce neuroblastoma cells to

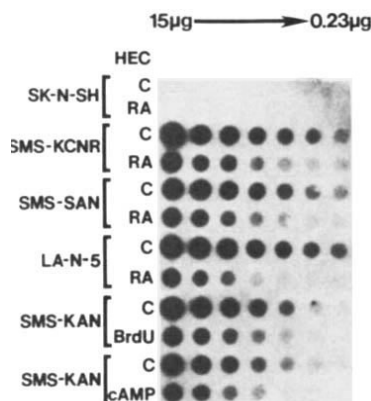


Fig. 3 Analysis of expressions of N-myc levels in cytoplasmic RNA (isolated as described in Fig. 2A) from four neuroblastoma cell lines (SK-N-SH, SMS-KCNR, SMS-SAN, LA-N-5) treated with solvent control (C) or retinoic acid (RA) and one neuroblastoma cell line (SMS-KAN) treated with either 6 μ M 5-bromodeoxyuridine (BrdU) or 1 mM dibutyryl-cyclicAMP (cAMP). RNA was isolated from cells when they showed maximal neurite outgrowth except for SK-N-SH which does not extend neurites in response to RA but does respond by assuming a more epithelioid morphology⁹: SK-N-SH+RA, 2 days; SMS-KCNR+RA, 10 days; SMS-SAN+RA, 7 days; LA-N-5+RA, 13 days; SMS-KAN+BrdU, 36 days; SMS-KAN+cyclicAMP, 12 days. RNA from a human embryonic fibroblast cell lines (HEC) was used as a control. RNA was denatured in 0.44 M formaldehyde and 3 $\times$ SSC at 60 °C for 15 min, chilled on ice and twofold serial dilutions were adjusted to 20 $\times$ SSC and applied to nitrocellulose sheets. Hybridization was performed in 50% formamide, 10% dextran sulphate, 5 $\times$ SSC at 42 °C using the ³²P-labelled pNb-1 DNA. Washing was done in 0.2 $\times$ SSC and 0.1% SDS at 60 °C.

acquire some morphological and biochemical properties of neurones⁶⁻¹¹. RNA was extracted from cells when they showed maximal morphological differentiation (as assessed by neurite outgrowth) and twofold serial dilutions of cytoplasmic RNA were immobilized on nitrocellulose and examined with ³²P-labelled pNb-1 DNA. The results of these RNA dot blots (Fig. 3) indicate that in all but one of the cell lines examined, an approximately 4–16-fold decrease in the level of N-myc expression was observed in the differentiated cells. The one cell line in which this did not occur was SK-N-SH which we found did not have detectable levels of N-myc before treatment and which has been reported not to contain amplified N-myc sequences⁴.

Studies on the induction of differentiation in several tumour cell lines, including teratocarcinoma^{14,17} and myeloid leukaemia^{13,18}, suggest that the genes required for normal growth and differentiation are not lost during tumour formation but that the regulation of these genes is altered in these tumour cells. Our experiments relate the expression of N-myc to *in vitro* morphological differentiation of human neuroblastoma cell lines and demonstrate that the level of expression of this amplified sequence is decreased in several cell lines induced to differentiate. This is consistent with previously described differential expression of proto-oncogenes during normal growth and development¹⁹ and suggests N-myc is expressed early in the development of neuroectodermal tissue. Our finding that N-myc levels decrease in RA-treated neuroblastoma cells before the onset of detectable morphological differentiation, yet are not depressed in growth-arrested cultures with similar cell-cycle profiles is compatible with the hypothesized regulatory activity for the N-myc gene product²⁰.

The presence of intracellular retinoic acid binding proteins in neuroblastoma²¹ suggests that the prompt regulation of N-myc could be mediated by a direct effect of RA, although other mechanisms such as RA-induced increases in cyclic AMP-dependent protein kinase activity²² cannot be ruled out. Since the levels of RA which induce neuroblastoma differentiation

are similar to levels that can be achieved clinically, RA itself may have therapeutic value in this disease.

We thank J. M. Bishop for providing pNb-1 DNA, Doug Feagans, Rene Parenteau and Donna-Maria Jones for their technical assistance, Debra A. Reynolds for the illustrations and Arlene Richardson for the preparation of this manuscript. This work was in part supported by Navy Medical Research Development Command, research work unit MR000.01.01.1300.

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- Kelly, K., Cochran, B. H., Stiles, C. D. & Leder, P. *Cell* **35**, 603–610 (1983).
- Campisi, J., Gray, H. E., Pardee, A. B., Dean, M. & Sonenshein, G. E. *Cell* **36**, 241–247 (1983).
- Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E. & Bishop, J. E. *Science* **224**, 1121–1124 (1984).
- Schwab, M. *et al.* *Nature* **305**, 245–248 (1983).
- MacMillan, R. W., Blanc, W. B. & Santulli, T. V. *J. Pediatr. Surg.* **3**, 161–164 (1968).
- Reynolds, C. P. & Perez-Polo, J. R. *J. Neurosci. Res.* **6**, 319–325 (1981).
- Prasad, K. N. & Kumar, S. *Cancer* **36**, 1338–1343 (1975).
- Sidell, N. *J. natn. Cancer. Inst.* **68**, 589–593 (1982).
- Sidell, N., Altman, A., Hassler, M. R. & Seeger, R. C. *Expl. Cell Res.* **148**, 21–30 (1983).
- Prasad, K. N., Mandal, B. & Kumers, S. *Proc. Soc. exp. Biol. Med.* **144**, 38–42 (1973).
- Spinelli, W., Sonnenfeld, K. H. & Ishii, D. N. *Cancer Res.* **42**, 5067–5073 (1982).
- Reynolds, C. P., Reynolds, D. A., Frenkel, E. P. & Smith, R. G. *Cancer Res.* **42**, 1331–1336 (1982).
- Breitman, T. R., Selonick, S. E. & Collins, S. N. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2936–2940 (1980).
- Strickland, S. & Mahdavi, V. *Cell* **15**, 393–403 (1978).
- Westin, E. H. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **79**, 2490–2494 (1982).
- Dalla-Favera, R. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **79**, 6497–6501 (1982).
- Hilmssee, K. & Mintz, B. *Proc. natn. Acad. Sci. U.S.A.* **73**, 549–551 (1976).
- Reitsma, P. H. *et al.* *Nature* **306**, 492–494 (1983).
- Muller, R., Slamon, D. J., Tremblay, J. M., Cline, M. J. & Verma, I. M. *Nature* **299**, 640–644 (1982).
- Lee, W. H., Murphree, A. L. & Benedict, W. F. *Nature* **309**, 458–460 (1984).
- Haussler, M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **80**, 5525–5529 (1983).
- Ludwig, K. W., Lowey, B. & Niles, R. M. *J. biol. Chem.* **255**, 5999–6002 (1980).
- Krishnan, A. *J. Cell Biol.* **66**, 188–192 (1975).
- Reynolds, C. P. & Maples, J. *Adv. Neuroblastoma Res.* (in the press).
- Barlogie, B. *et al.* *Cancer Res.* **36**, 1176–1180 (1976).
- Kohl, N. E. *et al.* *Cell* **35**, 359–367 (1983).
- Evans, A. E., Baum, E. & Chaud, R. *Arch. Dis. Child.* **56**, 271–274 (1981).
- Beckwith, J. B. & Perrin, E. V. *Am. J. Path.* **43**, 1089–1104 (1963).

Errata

Accelerator mass spectrometry radiocarbon ages of amino acid extracts from Californian palaeoindian skeletons

J. L. Bada, R. Gillespie, J. A. Gowlett & R. E. M. Hedges
Nature **312**, 442–444 (1984).

THIS letter was inadvertently printed before all the proof corrections had been received. The first sentence of the fifth paragraph should read 'The radiocarbon age obtained for the La Jolla Shores skeleton agrees with a previously determined 5,000–6,000 yr AMS age of this skeleton (T. W. Stafford, personal communication), whereas those obtained using the conventional radiocarbon method are much younger⁷.' Reference 23 should refer to the dates for the Sunnyvale skeleton, and the correct reference is: Taylor, R. E. *et al.*, *Science* **220**, 1271–1273 (1983). The fully corrected version is available in reprinted form.

South American modern beach sand and plate tectonics

P. E. Potter
Nature **311**, 645–648 (1984).

IN the title, 'South American' was incorrectly replaced by 'South African'.

Corrigendum Iridium in Mississippi River suspended matter and Gulf of Mexico sediment

F. D. Fenner & B. J. Presley
Nature **312**, 260–262 (1984).

THE value of iridium quoted for geochemical reference standard DTS-1 as 61 p.p.b. should read 0.67 p.p.b. (Gladney, E. S., Burns, C. E. & Roelandts, I. *Geostand. Newsl.* **7**, 3; 1982).

Seas of unreason

Hugh Trevor-Roper

Tödliche Wissenschaft: Die Aussonderung von Juden, Zigeunern und Geisteskranken 1933 – 1945.*

By Benno Müller-Hill.

Rowohlt Taschenbuch Verlag, Hamburger Str 17, Postfach 1349, D-2057 Reinbek bei Hamburg, FRG : 1984. Pp. 188. Pbk DM 10.80.

HITLER'S "Final Solution" of the Jewish Problem "solved" nothing. Rather, it raised new and bewildering problems for historians, philosophers and moralists. How could this vast operation of highly organized mass murder, which was not confined to Jews, come about? How could a civilized nation, in the twentieth century, not only permit it but actually co-operate in it? For by now all the old excuses and explanations have worn thin. That the German people did not know what was being done, that they had no responsibility for it, that it was the work of a criminal party which had usurped political power and set up a dictatorship — all this is now recognized to be a myth. The German people, or at least the German establishment, not only knew and tolerated what was being done — provided it was done out of their sight and hearing — but had prepared the ground and provided the means for it. It is chastening, and disturbing, to reflect how the matter would now stand if Hitler had won his war. How much would we then know about the fate of the Jews, the gypsies and the other "inferior" peoples who had disappeared? How much further would the process have gone? How curious, or contrite, would the German people be? Would a new generation be reconciled to a victory on such terms?

At least we now know the factual history. The machinery was first set up to serve the "Euthanasia" programme for the extermination of the mentally ill. It was transferred to the East after the invasion of Russia, then "improved" to cope with millions of victims, who now included Slavs and gypsies. It continued to operate throughout the war, even when the German armies were everywhere in retreat. The industry of destruction seemed, at times, to take precedence over the necessities of war. But to establish the facts is not to explain. At the highest level of command we can assume a fanatical racial ideology. Hitler and Himmler had convinced themselves and their followers that the destruction of the Jews was a necessary

act of disinfection: the extermination of biological parasites who would otherwise destroy the "superior" German race. But what of the technological élite who organized the chemistry and lubricated the process of destruction: the white-coated doctors, who selected the candidates for the gas-chamber, who experimented on the

IMAGE
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REASONS

Selection at Auschwitz. The man on the left is supposedly Mengele.

victims while they were still alive, and who dissected their bodies and sent their pickled organs for "research" in their institutes when they were dead? The worst of these — those who actually participated in the murder — did not escape retribution, although Dr Mengele still lives comfortably in Chile; but others, who merely used or blessed their labours, returned to their laboratories and lecture rooms and continued their studies and their teaching, as if innocent of any complicity. By what historical or psychological process, we ask, did these educated men, who professed a humane science, become essential agents in the most grisly actions of a now universally dishonoured regime?

The experts concerned were mainly geneticists, who accepted perversions of Darwinism; anthropologists, who believed in the irremediable and inherited inferiority of certain races; and psychiatrists, who believed in the inheritance of mental illness. Professor Müller-Hill, a geneticist at the University of Cologne, has been

exercised by the problem of the complicity of these men and has made a careful study of their work and personality. He has examined the documents, and has interviewed survivors and the families or assistants of those who have not survived. Many documents have been deliberately destroyed, and many of those whom he interviewed refused to allow the record of the interview to be published. The secondary sources on the subject are — no doubt for these reasons — very thin, and Professor Müller-Hill insists that much work still remains to be done. Nevertheless, he has built up a detailed history in order to show how, in Nazi Germany, the academic study and practice of anthropology and psychiatry "revealed itself, step by step, as mass-murder of nonconformists".

The central establishment in the story is the Kaiser-Wilhelm Institute in Berlin, and in particular its separate institutes for anthropology and psychiatry. This body was "the spear-head of scholarly advance" but already, by 1933, it had committed itself to racial doctrines. As the head of the Institute of Psychology, Professor Eugen Fischer, said to the new *Reichsarztführer* Dr Conti, "our eugenic movement has been going far longer than" the Nazi Party. So the new order was easily accepted. Within three months of Hitler's accession to power, the Institute obediently began the purge of its Jewish members. Thereafter it was plain sailing. First came the sterilization of the unfit. Here it was the psychiatrists who took the lead. Already the basic principles were laid down. Mental illness was incurable and hereditary. Science had proved

that. Therefore the victims had no rights; the doctors (with a judge) decided their fate. This work went on until the attack on Poland. Some 350,000–400,000 sterilizations had by then been carried out; but now the doctors were wanted for the war, "and also to kill those who previously would have been sterilized".

Throughout the war, the grandees of the Kaiser-Wilhelm Institute co-operated whole-heartedly with the Nazi Party. They organized crash-courses for SS doctors. They also received funds from SS sources for their research. Here too psychiatrists took the lead. "Neurotic" soldiers, they recalled, had thrown down their arms in 1918. That must not happen again. So soldiers who faltered at the front were stiffened by electric-shock treatment — "torture by electricity" which they would not wish to incur a second time. (If they resisted the treatment, they were judged "mentally ill" and in need of more of the same.) Meanwhile, at home, the

**Deadly Science: The Elimination of Jews, Gypsies and the Mentally Ill, 1933–1945.*

Euthanasia law was in force. The mentally ill could now be killed quietly. The victims were selected by the doctors and sent to special killing-stations, to be gassed. The Jews among them were sent to the new extermination camp at Chelmno in Poland. "The affair, which began in the greatest secrecy, was soon known to the whole German people." Lawyers, officials, clergy protested; but, says Professor Müller-Hill, "among all the letters, I have not seen one from a psychiatrist".

The "Euthanasia Action" was stopped in October 1941 and its machinery was turned against the Jews. That machinery included its minders, the psychiatrists. The doctors of the Kaiser-Wilhelm Institute can hardly have been surprised. Six months before, their leaders, Professors Fischer and Günther, had been guests of honour at a conference called by Alfred Rosenberg, Hitler's ideologist, on "The Total Solution of the Jewish Problem". The nature of the solution proposed had then been clear. But it was not only Jews who were now to be exterminated. Slavs also were genetically "inferior", so Russian prisoners-of-war were condemned to death by starvation or over-work. Professor Abel, of the Anthropological Institute, favoured "extermination of the Russian people", which however was thought impracticable. So was a plan — "historically justifiable on biological grounds" — to exterminate 15 million Poles. The gypsies, however, being fewer, were easier to deal with. They too had been scientifically studied. Dr Ritter, of the Racial Hygiene Research Department of the Reich Health Office, was in the process of preparing a special report on them.

In 1941 the gypsies had been included in the "Final Solution", and those of Russia and the Balkans were massacred *in situ*. Those of Germany had been deported to Poland in 1939. How were they now to be disposed of? A plan to drown them all in a ship in the Mediterranean was dropped for practical reasons. It was then decided to let them freeze to death. But Dr Ritter protested that he had not quite completed his research on them, which secured them another year of life. They then ended in the gas-chambers of Auschwitz. Those from East Prussia, who came separately, were pushed straight in on arrival by the considerate Dr Mengele, who was on the spot. He was afraid of an epidemic unless they were disposed of quickly. The extra year of life had not been useless to research: it had enabled Dr Ritter to confirm the assumptions from which he had set out. As Professor Fischer put it, "it is a rare and special good fortune for a theoretical science to flourish at a time when the reigning ideology welcomes it, and its findings can serve the policy of the state"; and Dr Ritter's conclusions about the gypsies could now provide a model for the treatment of other groups.

Professor Fischer had meanwhile retired from the Kaiser-Wilhelm Institute, but his

successor, Professor von Verschuer of Frankfurt, carried on the good work and, thanks to his assistant Dr Mengele, exploited "the gigantic opportunities" of Auschwitz. Dr Mengele supplied the Institute with "rare and valuable material" — human organs, pickled in spirits and sent by express post as "urgent war material". He also carried out projects of his own. Standing on the ramp at Auschwitz, he picked out twins, giants and dwarves and after experimenting on them alive, had them killed and dissected for further research. Other departments of the Institute also made use of the same opportunities. The Psychiatric Institute and the Brain Institute snapped up skulls as fast as they could be delivered. As the head of the Brain Institute, Professor Hallervorden, would explain afterwards to his American interrogator:

I heard that they were going to do this, so I went and said to them, 'Look here, folks, if you're going to kill all these people, at least take out the brains so that the stuff can be used'. They asked me, 'how many can you manage?', and I said, any number, 'the more the better'. I gave them fixing material, jars and boxes, and they turned up with them like a removal-firm with a furniture-van.

Unfortunately there could be hitches, as Professor Schneider at Heidelberg lamented: only half of the children's brains arrived in usable condition: "that is a pity", he wrote philosophically to his old colleague in the Euthanasia programme, Professor Nitzsche, "but it can't be helped".

At the end of his book, Professor Müller-Hill quotes a conversation between Dr Mengele and his Jewish "slave-assistant" at Auschwitz, Dr Nyiszli. "When will all this extermination stop?" asked the unfortunate prisoner. "My friend", answered his master, "it will go on, and on, and on". In fact it was stopped by the defeat of Germany; it is the questions that go on, and on. Professor Müller-Hill sums them up as "nine questions", which he does not presume to answer with confidence. He also gives — where it has been allowed — the text of his interviews. The fundamental question is, why did this happen only in Germany? Can it happen elsewhere? And the fundamental problem raised by the interviews lies in the fact that none of those professors who had participated — at a safe distance — in the grisly business, seems to have had any sense of personal responsibility for it.

Locked in their complacent, insular "objectivity", these men simply could not see that they could be accused of anything "unprofessional". And as they would not condemn themselves, so they would not

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Decompression experiment on an inmate at Dachau.

condemn each other. "I have asked all his surviving pupils whether Professor Fischer was an anti-semitic" writes Professor Müller-Hill. "No", they all replied, without exception: 'whatever else, he was not that'. And he was one of the Presidents of the Anti-Jewish Congress in Cracow! "How much Dr Mengele knew of the cruelty and murders in Auschwitz", wrote a committee of his fellow professors in 1949, does not emerge from the documents. "Let us say nothing of that: it all lies behind us", Fischer's successor Professor von Verschuer wrote calmly to a foreign scholar in 1946; and his own colleagues at least were prepared to agree: they would not be so "pharisaical", they said, as to judge a man who had all the qualities of an ideal researcher and teacher of youth. These men were a guild: "their colleagues were closer to them than their patients".

They also believed that they were "objective". They did not pervert their judgement by any ideological or moral prejudices. They were not Nazis. Professor Müller-Hill allows that "all of them — I exclude none — had mental areas which were not soiled by Nazism", "islands of rationality in a foaming sea of unreason". But if they were not Nazis, perhaps that was only because their prejudices, which they thought were pure science, preceded the Nazism which they willingly served. That, of course, only pushes the problem further back. Its solution must be sought not in the public history of Nazism but in the dark, still only half explored mental pre-history of that sinister movement. □

Hugh Trevor-Roper (Lord Dacre of Glanton) is Master of Peterhouse, Cambridge. He is a historian and author or editor of a number of books, among them The Goebbels Diaries, Final Entries 1945 (1978) and The Last Days of Hitler (6th Edn, 1982).

Philosophies and biologies

A.J. Cain

T.H. Huxley's Place in Natural Science.

By Mario A. di Gregorio.

Yale University Press: 1985. Pp. 247.

\$25, £21.

THE title of this book is somewhat misleading. Dr di Gregorio has indeed read Huxley's scientific papers, but, almost without exception, with no insight into their scientific content, the materials Huxley had to work with or the actual methods of a practising scientist. There are many doubtful statements on scientific matters (as well as a number of bad misspellings of scientific names) — *Macrauchenia* is not a camelid, a phylogeny of the Podophthalmia is not of the Crustacea (a far larger group), the "ordo Placoids" is a solecism and Haeckel was well aware of exceptions to recapitulation, to name only a few. It is incredible that (p. 97) "the evolution of groups above the level of species" can be described without qualification as "a kind of evolution which is still somewhat controversial", and even more so that this latter statement can be supported by a reference to William Paley's *Natural Theology* (1802).

What Dr di Gregorio is mainly concerned with is to extract from Huxley's scientific writings an analysis of his philosophy of science, and the psychological peculiarities that gave him his immense drive and success. Unfortunately part at least of Dr di Gregorio's conclusions depend on misreadings of nineteenth-century English prose, both scientific and informal. He believes that Huxley "over-read" Darwin's analogy of artificial and natural selection into a belief that they were "essentially identical" in action. A look at the use of analogy in the nineteenth century (and previously) would correct this impression. Even the eccentric William Swainson understood the difference between a superficial (or poetical) analogy and a real scientific one (see, for example, Chapter 6 of his *Preliminary Discourse on Natural History* of 1834).

Similarly a letter from Darwin to Huxley is exaggerated into a quarrel between them, and (with others) into a fundamental difference in their philosophies of science, Huxley requiring theory to be verified absolutely by direct experiment, Darwin

looking only to its powers of explanation. The real issue here is that Darwin (a good experimentalist) believed like most people that evolution was so slow that an experiment to produce a new species would take far too long; Huxley, presumably influenced by the advances in livestock breeding of the previous half-century, thought it must be successfully carried out before evolution could be more than the best hypothesis so far. Dr di Gregorio quotes (p. 62) from a letter to Hooker, Darwin's statement that "the change of species cannot be directly proved" but exalts it into a major difference in philosophy instead of a practical difficulty.

Not realizing the practical value of Huxley's use of the type concept, Dr di Gregorio accuses him of employing a metaphysical term without a proper system of philosophy, and again mystifies the situation because he has no experience of its employment empirically both in grasping the diversity within a group and in teaching it. Viewing Huxley primarily as a careerist driven by a sense of insecurity, he sometimes praises him for his fairness and grasp of the subject. More often he seems to regard him as a fool, a crafty opportunist and a mere power-seeker. The best thing that this book can do is to stimulate people to read Huxley and judge for themselves. □

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Key to symmetry

J.P. Elliott

Group Theory in Physics, Vols 1 and 2.

By J.F. Cornwell.

Academic: 1984. Vol. 1 pp. 371, \$75, £49.50. Vol. 2 pp. 562, \$100, £70.

If mathematics is the tool-chest of the theoretical physicist, then group theory is the instrument which opens up an understanding of symmetries. Of course, group theory is only algebra, but it would be foolish today to argue that one should carry out the algebraic steps without recognizing the group-theoretical structure of those processes.

Although group theory plays a role in classical physics, it is in the realm of quantum physics that it dominates. In some concrete applications, vibrations for example, group theory can be seen as a neat way of understanding results that would emerge, in any case, from numerical calculations. However, in more abstract fields such as elementary particle physics, where the new concepts of strangeness, charm and colour, and even the particles (quarks) themselves are quite beyond human experience, symmetry often provides the foundation of the theory. Group theory can also be seen as a unifying element enabling us to recognize, as

consequences of the same symmetry arguments, certain features such as degeneracies and selection rules which occur in diverse physical systems.

It is appropriate therefore that the seventh title in the *Techniques of Physics* series should be *Group Theory in Physics*. Many books have been written on this topic since the 1920s but, even in 900 pages, an author faces some difficult decisions. First, should he concentrate on just one branch of physics, to allow space to describe both the physics and the group theory, or should he stress the wide range of application of group theory by describing many fields with only a brief account of the relevant physics? Secondly, what balance should be struck between readability and mathematical rigour? And finally, for whom is the book to be intended?

On the first question, J.F. Cornwell chooses to cover several fields and the 900 pages in two volumes are allocated roughly as follows: molecular and solid-state physics (100), elementary particle physics (90), Schrödinger equation (30), Lorentz groups (50), general group theory (100), Lie groups and algebras (300) and appendices (200). Atomic and nuclear structure problems get little or no mention. On the second question, the books emphasize mathematical rigour more than most of their competitors making it rather difficult for a reader meeting these topics for the first time. For example, molecular vibrations are discussed through a succession of eight theorems with proofs, and generally the style is that of a mathematics rather than a physics textbook. The third question is not answered in the preface, but the principal target must be postgraduate students getting to grips with theory in solid-state or elementary particle physics.

The strength of these books lies in the extensive account of Lie groups and their relation to Lie algebras, here done more simply than in a genuine mathematics text. This makes them a useful reference work for people who are already familiar with the basic ideas. On more detailed points I was surprised to find the symmetric group of permutations discussed, not in its own right, but as part of the study of the unitary groups. Also, having seen a mention of super-algebras in the preface, I was disappointed to find no further discussion of this comparatively new development except for an "aside" on one other page.

Technically, the two volumes are nicely produced and even the most elaborate equation is easy to read, though cross-reference by chapter and section is difficult because only the page number is carried at the top of each page. So one has to refer to the contents every time. Special praise, however, for the proof-reading. There must be misprints in a work of this length, but I didn't find any! □

J.P. Elliott is Professor of Theoretical Physics at the University of Sussex.

New in paperback

● *Alan Turing: The Enigma of Intelligence* by Andrew Hodges. Publisher is Unwin Paperbacks (Counterpoint imprint), price is £5.95. For review see *Nature* 307, 663 (1984).

● *Charles Babbage: Pioneer of the Computer* by Anthony Hyman. Publisher is Oxford University Press, price is £4.95. For review see *Nature* 300, 114 (1982).

Conscious beyond ourselves

Stuart Sutherland

Animal Thinking.

By Donald R. Griffin.

Harvard University Press: 1984. Pp. 237. \$19.25, £15.50.

THE ascription of consciousness to other beings surely depends on their similarity to ourselves, and in particular on how far their activities resemble those of our own that are consciously conducted. The relevant similarities are, however, not merely those of behaviour. Most people would be reluctant to think of a robot as conscious no matter how purposeful its activities, so long as its body was made from metal plates and its brain and nervous system from electronic components. Indeed, when it comes to consciousness, it is doubtful whether there can be any substitute for flesh and blood. For these reasons, most people would have no hesitation in thinking of other primates as conscious and would probably extend the attribute to many other mammals. There is no clear point in the animal kingdom where it is possible to be sure that consciousness stops, though paramecia and even ants are so different from ourselves that if they have some form of consciousness one feels it is likely to be very different from our own both in degree and in kind. It is of course equally difficult to decide at what point the growing fetus develops consciousness: to judge from a baby's undifferentiated behaviour, it may be many months after birth before it achieves the sort of awareness an adult has.

In *Animal Thinking*, a coda to his previous book *The Question of Animal Awareness* (second edition, 1981), Donald Griffin suggests that virtually all animals with a nervous system are conscious. He does not define consciousness since, as he rightly observes, such definitions are circular involving the use of terms like intention, idea, notion, awareness and so on. In trying to decide how far down the animal kingdom consciousness extends, he appears to use three criteria.

The first and most compelling is that an animal should exhibit "versatile adaptability of behaviour to changing circumstances and challenges". The most enjoyable and instructive parts of the book are those in which Griffin demonstrates the existence of this kind of behaviour by describing such activities as plovers drawing a predator away from their nests, ant-lions digging pits for prey and throwing sand at them, the construction of "house" cases by the larvae of caddis flies, and the recently learned ability of dolphins to escape from the nets used in tuna fishing. To the argument that such activities as the dance of the honey bee are largely genetic-

ally determined, he replies rather weakly that even so they may involve an element of consciousness.

The word "may" occurs repeatedly throughout the book, particularly in connection with Griffin's second argument, which is that whenever an animal performs a complex activity, it might be useful for it to be conscious of what it is doing. In fact, this is less an argument than an assertion. As far as we know, there is no behaviour, no matter how complex, that could not be exhibited by an organism or by a computer without consciousness, and many of the unconscious calculations made in perception or in controlling motor activity appear to be as complex as the thinking of an Einstein.

Griffin draws his third argument from Nicholas Humphrey, who has suggested that to deal with their co-specifics social animals must empathize with one another. But again, empathy is simply using a model of oneself appropriately modified to fit someone else's circumstances in order to predict his goals, needs and behaviour.

There is no obvious reason why such a model should be of use only if it appears in conscious awareness.

Griffin repeatedly urges that further research should be undertaken on animal consciousness, but he gives no indication of how to go about it. He also believes that only by understanding the consciousness of animals will it be possible to explain their behaviour. He forgets, however, that explanations in terms of consciousness are not rigorous since they rely on our manipulating a model of ourselves. To offer rigorous explanations it is necessary to specify the processes that underlie both a given sequence of thoughts and the organism's behaviour. It is worth asking whether the knowledge that a given species was conscious would really make any difference to how its behaviour is to be explained. The question is more difficult to answer than Griffin allows. □

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Resolution to do better

Akiyoshi Wada

Methods of Protein and Nucleic Acid Research, Vols 1 and 2.

By Lev A. Osterman.

Springer-Verlag: 1984. Vol. 1 pp. 342, DM156, \$59. Vol. 2 pp. 204, DM98, \$38.

IT IS difficult to over-estimate the contribution made by spectroscopic methods to the advancement of modern physics and chemistry during the past one hundred years. Electrophoresis and ultracentrifugation, the topics covered in Lev A. Osterman's two-volume work, are two of the most important methods of molecular separation and analysis, and are today playing analogous roles in the development of molecular biology and biophysics.

Just as the essence of spectroscopy is the separation of photons according to their energies, the essence of the molecular techniques is to distinguish between molecules according to their physical and chemical characteristics. The results obtained from the two sets of methods are also similar — one a set of bands on a photographic plate, representing molecular species, the other a series of peaks on recording paper showing the intensity of electromagnetic waves. In both cases the resolving power is a crucial factor in determining the quality of the result.

A further aspect of methods of molecular separation is their multidimensional nature. Any one of a variety of parameters influencing the migration velocity of molecules in the separation medium may be used — size, amount and sign of the electric

charge, buoyant density, affinity to a specific ligand. Therefore, a proper selection of the dimension of separation, as well as the search for new parameters to distinguish hitherto inseparable molecular species, are matters of much interest to those working on biological macromolecules.

Osterman's manual of research methodology gives exhaustive descriptions of electrophoresis, isoelectric focusing and ultracentrifugation (Vol. 1), and immunoelectrophoresis and the application of radioisotopes (Vol. 2). The two books should be extremely useful even to novice researchers; thorough and systematic description of the techniques and plentiful illustrations should enable both graduate students and more experienced researchers to perform high-resolution experiments with the most suitable dimensions of separation. The coverage of the techniques and the references are comprehensive and current to the end of 1982.

The books should also help in the choice of the most suitable combination of the various separation techniques to obtain results which could not be obtained with one alone, for example two-dimensional display by O'Farrell's method. The quality of the results emerging from such experiments ultimately depends on the individual scientist's skill and training, but these two volumes provide an excellent starting point.

I have one plea. Various types of chromatography are equally important to macromolecular fractionation. In a future edition Dr Osterman might consider including them to make his manual even more complete and valuable. □

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The borrowers afield

Peter J. Bowler

Darwinian Evolution.

By Antony Flew.

Paladin: 1984. Pp. 149. Pbk £2.50.

THE prospectus for Paladin's *Movements and Ideas* series openly challenges Fontana's *Modern Masters* and Oxford University Press's *Past Masters*, arguing that major intellectual movements cannot adequately be described through the medium of biography. Antony Flew's *Darwinian Evolution*, one of the early volumes in the series, is thus meant to supplant the accounts of Darwin's work by Wilma George (Fontana) and Jonathan Howard (Oxford). *The three books have a similar format — all are slim paperbacks with peculiar covers — but their purposes are indeed rather different. Of the two earlier accounts, George's is more sympathetic to the cultural environment in which Darwinism developed, and makes an effort to relate the theory to wider debates. Flew's real interest is the latter area. He summarizes Darwin's career and the argument of the *Origin of Species*, but the substance of the book is its analysis of the philosophical and ideological issues.

It is perhaps inevitable that so brief a study should concentrate on the areas with which the author is most familiar, but some readers may find the choice of topics unbalanced. Flew's purpose is to uncover the logical core of Darwinism and then show how that core fails to support many of the ideological systems that have used it as a foundation. He explores the logical structure of the theory of natural selection, and refutes at length the claim that the theory rests on a tautology. In the process, the cladists at the Natural History Museum are dismissed as Aristotelians. But we are told virtually nothing about taxonomy, biogeography, speciation and other topics of primary importance to Darwin and all later Darwinians. The structure of the theory is obviously important, but I doubt that a newcomer to the field can be given an adequate impression of what scientific Darwinism is all about by concentrating so narrowly on this topic.

On the ideological front, Flew discusses the influence of Malthus and Adam Smith at some length. He defends Malthus against the criticisms of Marx and other social reformers by stressing the deductive character of the principle of population. Curiously, the movement to defend *laissez-faire* capitalism as an extension of Darwinian principles is passed over in a couple of pages, and Herbert Spencer receives scarcely a mention. Flew's energy is conserved for an assault on the Marxists, whose links with Darwinism are systematically demolished. His arguments are

valid, but the section degenerates into a wholesale critique of Marxism which seems out of place in a book supposedly devoted to Darwinism. Flew also attacks Desmond Morris and those who would reduce human beings to animals, but defends sociobiology against intolerant radicals by pointing out that it is not meant to explain all human behaviour.

Flew's efforts to expose the weak links in the ideological connections are stimulating contributions to a number of debates, but

in a series entitled *Movements and Ideas* one might have expected a more historical approach. The various forms of social Darwinism may be perversions of the scientific theory, but we need to know why so many political movements have tried to link themselves with Darwin's name. □

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Price changes

Michael F. Claridge

Insect Ecology, 2nd Edn.

By Peter W. Price.

Wiley: 1984. Pp. 607. \$47.50, £37.50.

THE second edition of Peter Price's *Insect Ecology*, the first of which was published in 1975, is far more than an up-dated and corrected reprint. Undoubtedly the most significant feature of the first edition was the stress placed upon the evolutionary aspects of the subject. This emphasis is retained, but thorough revision has produced a more balanced text. The book deals with the fundamental problems of ecology as exemplified by terrestrial ecosystems. Such ecosystems are mostly dominated numerically by green plants and insects, but while the author is thus able to consider all of the major aspects of terrestrial ecology by reference to these organisms, he frequently uses examples from other taxa and habitat types when they are relevant to his main theme.

The introductory chapters provide a brief but excellent account of the principal components and the functioning of ecosystems. A new chapter is included on the very important ecological consequences resulting from the relatively small sizes of insects. Most of the remainder of the book consists of chapters under three general headings — "Trophic Relationships", "Populations" and "Communities and Distributions" — which develop in a more logical and satisfying manner than in the first edition. There are useful accounts of insect-plant, predator-prey and parasite-host interactions, while elsewhere Price argues persuasively for the importance of mutualistic interactions which he justifiably claims have been sadly neglected until recently by ecologists. He also provides a stimulating and thought-provoking discussion of the possible evolutionary consequences of different types of interspecific interactions.

The chapters concerned with population processes have been extended, and in themselves constitute a helpful text for students. The final section, on community ecology, includes lucid accounts of some current areas of ecological controversy, such as the role of competition in the structuring of

communities and the relationships between diversity and stability.

For this new edition the original material has obviously been reworked in detail. In most areas this has led to a clearer and better book. For example, a whole chapter was previously devoted to "species packing"; now the term does not even appear in the index, and the phenomena are dealt with more objectively under the niche and related concepts. Rightly so — ecology does not need redundant terminology!

The book is well written, as before, and is illustrated with many new and beautiful line drawings. In its new, improved form, *Insect Ecology* will continue to be the place to start for advanced students of the subject. □

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* Reviewed together in *Nature* 296, 510 (1982).

A man with qualities

John C. Marshall

Robert Musil and the Culture of Vienna.
By Hannah Hickman.
*Croom Helm, London/Open Court
Publishing, La Salle, Illinois: 1984.*
Pp. 203. £15.95, \$24.95.

THAT doctors (neurologists and psychiatrists in particular) should write fiction of compelling interest does not strike one as particularly surprising. Their working lives as secular priests allow them to poke around in corners of the body and soul that we usually try to keep well hidden from even our most intimate friends, and the "raw material" thus uncovered has supplied themes aplenty for such doctor-writers as Arthur Schnitzler, Ernst Weiss, Anton Chekhov, Géza Csáth and Alfred Döblin. Furthermore, the well-constructed case-report that young doctors are trained to present on ward-rounds ("On examination, . . .") is itself a literary form of considerable power. Whatever one may think of them as "science", Sigmund Freud's early papers have the shape and elegance of superbly crafted short-stories. And Freud indeed once wrote to Schnitzler, a master of the short-story form, that he had always avoided him for fear of meeting his *Doppelgänger*.

That training as an experimental psychologist could yield a comparable input to the creative imagination sounds, at first blush, simply ridiculous. The discipline prides itself on providing "shallow" interpretations of "objective" behaviour elicited under carefully controlled conditions and subjected to rigorous methodological and statistical scrutiny. What could possibly be further from the lush profligacy of both fictional and "real" life? Yet it is exactly such a background, combined with the hard sciences and engineering, that provided the motive forces behind the only truly "modern" novel written in the twentieth century: Robert Musil's ironic dissection of the Austro-Hungarian Empire as it floated towards destruction in the First World War — *The Man without Qualities*. Many of us who misspent our youth reading Musil when we should have been studying for examinations will welcome Hannah Hickman's summary of Musil's life and work, and her analysis of the influences and experiences that made him the outstanding chronicler of the culture of Vienna in the Gay Apocalypse.

Robert Musil (1880–1942) spent his childhood and adolescence in Austrian military academies until in 1898, at odds with the intellectual and cultural values of army officers, he entered his father's department at the Technical University in Brno, Moravia, to train as a mechanical engineer. After a brief period as an assistant at the Technical University in Stuttgart,

dissatisfaction with engineering as a philosophy of life provoked Musil to re-enroll as a student, this time at the University of Berlin. There he read logic, mathematics, physics and experimental psychology. Musil's professor was Carl Stumpf who had made important contributions to the study of auditory perception and to the psychology of music: Stumpf was also instrumental in unmasking "Clever Hans", the mathematical horse. Other students in the department in the early years of the century included Wolfgang Köhler, Max Wertheimer and Kurt Koffka, for whom Stumpf's stress on intrinsic perceptual organization was a crucial factor in their development of *Gestalt* psychology. Musil himself performed tachistoscopic experiments and devised a greatly improved colour wheel that became standard apparatus in all European laboratories of psychology. For his doctoral dissertation, however, Musil wrote an analysis of Ernst Mach's psychophysical theories. Mach's impressionistic materialism was not congenial to the department and "it was only after repeated arguments", Hickman recounts, "that Stumpf could be persuaded to accept Musil's thesis".

Both strands of Musil's psychological education emerged in his masterpiece. Ulrich — the man without qualities — lives through the first half of the novel as a fragmented empiricist, sardonically observing Franz Josef's reign as "Emperor of Peace"; but in the second half he attempts to recreate, in a potentially

incestuous relationship with his sister, the vision of wholeness that informed the work of the *Gestalt* psychologists.

Although brief, Hannah Hickman's monograph gives a comprehensive account of Musil's writings, from his first novel (*Young Törless*) to his (unsuccessful) efforts to complete *The Man without Qualities* while in Swiss exile after the *Anschluss*. She draws extensively upon the published notebooks and letters and the many unpublished papers in the National Library of Austria; her wide reading is reflected in the particularly skilful way in which she interrelates Musil's biography, essays and fiction. Only a fraction of Musil's output has been translated into English (though his finest work, *The Man without Qualities*, is still in print). For the reader with little or no German, Hickman's study brilliantly fills out the background that will deepen one's appreciation of the major fiction.

The monograph will undoubtedly send the reader back to Musil with an increased awareness of the *psyche* of a writer who could employ such chapter titles as "From Koniatowski's critique of Danielli's theorem to the Fall of Man"; "So far as jurists are concerned, there are no semi-insane people"; and, my own favourite, "A chapter that can be skipped by anyone who has no very high opinion of thinking as an occupation". □

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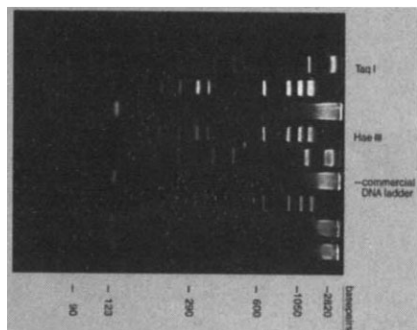
In business — jubilation in space, November 1982, as the Space Shuttle project starts to pay off with the successful launch of two commercial communications satellites from Columbia. The picture is reproduced from The Voyages of Columbia: The First True Spaceship by Richard S. Lewis, published (appropriately) by Columbia University Press. Price is \$24.95, £20.80.

Chromatography and electrophoresis

A wide variety of new products is covered, including several chromatography data handling systems and ten offers of free literature or applications notes.

● NuSieve agarose is a new gel medium designed for sieving low molecular weight DNA. Produced by the Marine Colloids Division of FMC Corporation, NuSieve agarose features low viscosity to allow simple casting of high concentration gels. The gels are firm and easy to work with, and this one-component gel is a convenient alternative to composites of agarose and polyacrylamide gels. NuSieve agarose will separate DNA fragments in the 10–1,000 basepair range, making it suitable for checking the quality of DNA linkers.

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NuSieve in action.

● A new range of higher purity solvents has been introduced by BDH to meet the demands in HPLC for ever-higher purity solvents as the technique provides greater sensitivity. The HiPerSolv range of solvents is extremely pure and suitable for the most sensitive HPLC work. The solvents also have minimal water content, low non-volatile matter content and high optical purity. The HiPerSolv range comprises sixteen high purity solvents, available in 2.5 and 1.0 litre packs.

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● The Packard Instrument Model 436 gas chromatograph is an economical instrument which is capable of superior performance in routine capillary column separations. Features include idealized oven geometry, microprocessor control, high-speed electrometers, proportional oven temperature control, high quality insulation, a non-turbulent column environment and precise flow control. Model 436 is ready to accept microbore column technology without need for modifications, a feature that will become increasingly important as these new columns come into regular use.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.

● Erba Instruments has developed an automated liquid injection system for completely unattended cold on-column injection of up to 60 samples, the Autosampler 550. The new autosampler is extremely compact and can be added to the Erba "Mega Series" HRGC system.

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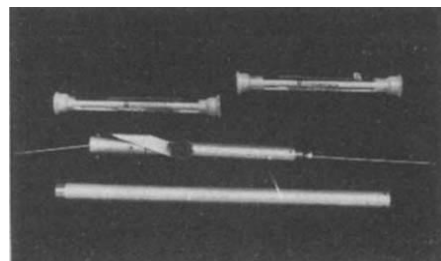
Hewlett Packard's new liquid chromatograph.

● High-speed chromatography is combined with high resolution in the new IBM 3 μ m Laureate Series HPLC columns. These columns come packed with a proprietary 3- μ m spherical silica and are available in silica and in two bonded phases — octadecyl and octyl-EC (encapped). These 4.5-mm internal diameter 3- μ m columns are offered in two column lengths, 50 mm and 100 mm. Analytical methods can be easily transferred from 5- μ m columns because the chemistries of the 5- μ m and 3- μ m columns are identical.

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● Three new models have been introduced to Hewlett Packard's HP1090 family of integrated LC modules. Now the HP1090 can be controlled from its own keyboard for applications which do not require the computing power of the HP85 controller. The Local User Interface, or LUSI, provides a lower entry price to the HP1090 family without compromising on chromatographic performance. Up to 10 LC methods (parameters for solvent delivery, sample injection and detection) can be stored in a battery protected memory and parameters may be changed instantly during a run. For outstanding composition precision and an economical solution to ternary mixing and gradient operation, the new PV5 Ternary Solvent Delivery Module can be used with standard, high-speed and microbore columns. The third new module, the DPU multichannel integrator, quantitates up to eight signals from the HP1090 diode array detector module simultaneously. In one run, the integrator gathers and evaluates all the useful chromatographic data.

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Chrompack columns for separating organic acids and carbohydrates.

● Based on a microporous sulphonated polystyrene-divinylbenzene resin, Chrompack's new polymeric columns are designed for the separation of organic acids and carbohydrates. The columns for separating organic acids and aromatic acids are in hydrogen form, and the column for separation of carbohydrates is in calcium form. Dimensions are 300 mm long and 6.5 mm internal diameter for the organic acids and carbohydrate columns, and 100 $\times$ 6.5 mm for the aromatic acids column. The principal separation mechanism in these columns is ion-exclusion, but other mechanisms are observed due to the interaction between the non-polar groups present in the solute.

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● Pierce Chemical Company has introduced Excellulose GF-5, a pre-swollen gel filtration support for desalting solutions containing macromolecules. Excellulose GF-5 is a cross-linked beaded cellulose of with an exclusion limit of 5,000 daltons. Macromolecules, with a molecular weight greater than the desalting gel's exclusion limit, pass around the beads and are eluted in the column's void volume. Smaller molecules penetrate the pores of Excellulose beads and are eluted in one column volume. Excellulose GF-5 may be reused after the low-molecular weight compounds have been removed. Excellulose GF-5 can also fractionate small molecules. Polyethylene glycols, with a molecular weight of 400 to 5,000 have been successfully separated using this support.

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● Alltech Associates' line of 0.53-mm fused silica NON-PAKD GC columns is claimed to offer capillary column convenience coupled with an improved performance on most applications previously the province of packed GC columns. The columns are extremely inert and are claimed to give equal or better separations than packed columns while running 3 to 5 times faster and with significantly reduced peak tailing.

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● LDC/Milton Roy's modular ion chromatography system, featuring the new conductoMonitor III conductivity detector is an ion chromatography system that routinely delivers low p.p.b. (parts per 10⁹) detection of common anions and cations and includes a Rheodyne sample injection valve and all necessary plumbing for simple and quick system set-up. It features a wide zero suppression range to electronically suppress the background conductivity of eluent buffers and cell temperature is actively controlled for baseline stability.

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● A new line of ampholytes has been developed by Isolabs to meet the demand for high resolving, stable, pH gradients within electrophoresis gels. Isolytes are produced in eleven pH ranges, and resolve molecules with isoelectric points differing by as little as 0.02 and have excellent buffering capacity at each pI, with uniform conductivity. To assist with the observation of when the gel has equilibrated a light amber colour has been introduced which enables the Isolytes to be observed as they migrate to the pI position.

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● A compact, easily fitted densitometer accessory is now available with the new Pye Unicam PU 8800 UV/visible spectrophotometer series, making the instrument ideal for recording electrophoretic separations. The old technique of staining and then photographing the gel suffers from a loss of detail, and the new accessory converts the PU 8800 intelligent, high-speed UV/visible spectrophotometer into a high-performance densitometer for this increasingly popular method of following electrophoresis.

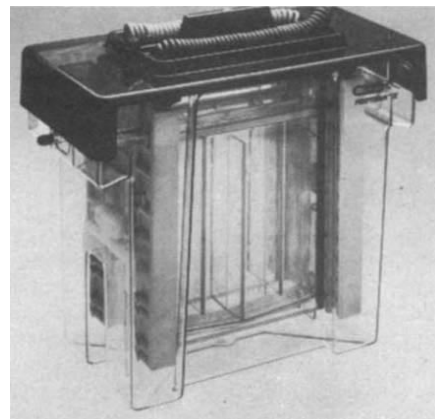
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● The Eldex column heater is designed to improve chromatographic precision by providing a stable, temperature controlled environment. The heater incorporates precise temperature control up to 150°C, an optional heated injection valve, and can accommodate two columns. It will accommodate up to two standard 30 cm columns of up to 0.375 inches outer diameter, plus guard columns. Many different sized columns can be used in the same heater, as the aluminium inserts are interchangeable.

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● The capabilities of the Perkin-Elmer Model 8300 gas chromatograph have been extended with major new options and an uprated specification. The Programmable Temperature Vapourizer (PTV) is a temperature programmable injector that provides both split and splitless operation. Samples are injected into a cold environment so that problems associated with sample discrimination from the syringe needle, decomposition of labile compounds and quantitation errors are greatly reduced. In many applications where on-column injection has previously been required, excellent results can be obtained with the PTV. The Model 8300 real-time screen graphics and built-in data handling facility provides a versatile chromatography and data processing system, and a new option adds re-integration of raw data to this system. Following an analysis, the chromatogram can now be re-examined, integration parameters changed, recalibrations carried out and, if necessary, the process repeated until the most suitable parameters are determined.

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The Protean II electrophoresis cell.

● Protean II is a vertical electrophoresis system from Bio-Rad, designed for easy operation for SDS-PAGE, two-dimensional and many other techniques. The Protean II system comprises: vertical multi-tube and Trans-Blot cells; gradient formers, slab dryers, destainers and power supplies. The Protean II vertical cell is easy to assemble, requires no grease and is leak-proof. Smile-free patterns are obtainable with only 1.5 litres of buffer and up to four 16 × 16 cm or 16 × 20 cm slabs can be run simultaneously. For larger capacities the multi-cell can accommodate up to 12 slabs or 48 tube gels.

Circle No. 115 on Reader Service Card.

● New medium-pressure packings, made of spherical, beaded cellulose, are now available from Amicon for ion-exchange and gel permeation chromatography. With outstanding physical strength and pressure resistance as well as low nonspecific adsorption, these media are specially suitable for high column flow rates. The rigidity of the particles in the pre-swollen gels allows repeated repacking and stirring without generation of fines. Large bed height changes due to changes in solvent conditions are avoided, as a result of rigid structure. Offered under the trade name Matrex Cellufine, the line of media includes medium and high-MW ion-exchange and desalting gels.

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● Automated Microbiology Systems, Inc. has developed a new scanner capable of scanning gels, TLC, and paper chromatograms labelled with ¹⁴C, ³⁵S, or ³²P. The Ambis Beta scanning system features a two-dimensional proportional counter with 312 discrete detector elements. This detector array scans an entire gel or TLC plate to produce an image of the distribution of activity with up to 47,000 image points, allowing the user to select regions of interest. Data from these regions can be processed and presented as histograms. The software facilitates easy quantitation of activity within the peaks as well as peak location. The system is controlled by a microcomputer that automatically performs the entire scanning procedure.

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● For milligram to multigram separations, the Separations Technology/Lab 800A preparative HPLC system has been engineered to facilitate the use of smaller particle packing media for increased separation efficiency. Each system is equipped with a solvent delivery system, a fluid control panel, an ST/2000 series stainless steel column and a mobile cart with a column mounting assembly. Dual-head diaphragm type pumps deliver a maximum flow rate of 800 ml per min at 1,800 p.s.i. for either isocratic or binary gradient solvent applications. Systems are provided complete with detection, recording and fraction collection ability. Pilot-plant and process scale systems are also available.

Circle No. 114.

● Nelson Analytical's Series 4400X chromatography data systems are based on a new power-packed software package called XtraChrom. XtraChrom offers graphic-aided on-screen editing, a post-run processing routine for recalculation, comparison and ratioing, and real-time integration. The Series 4400X system is based on the Hewlett-Packard Series 200 technical desktop computer. Nelson Analytical develops the chromatography software for the HP computer — a turnkey system specifically tailored for chromatography data handling and reduction.

Circle No. 118 on Reader Service Card.

● Chromatochart, a new software package from Heyden Datasystems, uses the Apple II microcomputer to achieve multi-channel chromatography data handling. The combination permits users to acquire, analyse and report chromatographic data which can be fed in from capillary and packed column gas chromatographs as well as HPLC units, gel scanners, TLC densitometers and amino acid analysers. The system is user friendly, with procedures for data acquisition, display, storage and output routines all menu driven. Data acquisition and integration parameters can be defined, stored, and edited as required. Powerful integration routines measure peak area, retention time, height, width at half-height and skew.

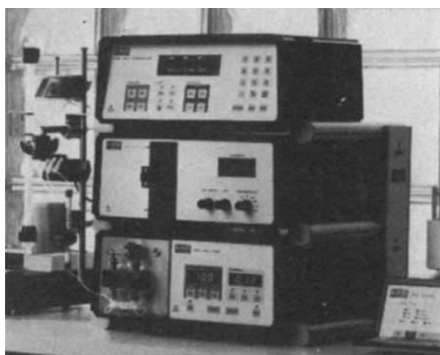
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● A new range of chromatographic columns, Zorbax BioSeries, designed for separations of compounds of biological origin and interest, has been developed by Du Pont, and the first two products in the BioSeries range are now available. These are the Zorbax BioSeries PEP/RPI column for the reversed phase separation of peptides, proteins and digests, and the Zorbax BioSeries GF250 for high performance gel filtration separations of peptides and proteins. The latter is based on a new zirconia-stabilized silica which gives superior stability at higher pH values than other columns. Other columns in the BioSeries, including a PTH column for the isocratic separation of PTH amino acids from gas phase sequencing, are to be announced shortly.

Circle No. 120 on Reader Service Card.

● The Pharmalyte series from Pharmacia Fine Chemicals has been extended with the introduction of five new narrow pH intervals for isoelectric focusing and one wide interval for two-dimensional electrophoresis. The series now covers the pH range in which the majority of proteins have their pIs. The new narrow pH interval Pharmalytes (4.2–4.9, 4.5–5.4, 5–6, 6.7–7.7 and 6–8) have a larger number of carrier ampholyte species for high resolution. The Pharmalytes generate uniform, linear pH gradients, with low and even conductivity and high buffering capacity for stable focusing at up to 3,000 V.

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For the scale-up of separation procedures, the LKB 2237.

● LKB-Produkter AB has introduced a completely modular line of preparative scale columns and accessories designed to further simplify the scale-up of chromatographic separations. LKB's 2237 preparative-scale columns and accessories enable the user to custom-design an ideal preparatory or production-level system to handle working volumes of aqueous or organic solutions in the 0.2 to 10 litre range. Two series of columns are available — the Aqueous Solvent Series and the Organic Solvent Series. They enable all of the buffers and solvents required for even the most complex purification schemes, such as those for certain peptides, hormones and lipids, to be used. LKB 2237 columns are available in two diameters (5 and 10 cm) and three lengths (60, 100 and 120 cm).

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● A new low-cost Chromatography Data Station based on the BBC microcomputer is now available from Jones Chromatography. The Menu-driven software package permits even the most inexperienced user to obtain results and reports rapidly using single-key operation. The facilities provided include data acquisition and storage, screen display of the real time chromatogram, calculation of peak area, peak height, area % and retention time. Both chromatogram and results can be viewed on the screen before committing to print on the 4-colour printer-plotter. Complete chromatograms can be stored on the disk system. The system comes complete with 32K BBC microcomputer (model B), 100K disk system, 9-inch green screen monitor, printer-plotter, chromatograph interface, all necessary cabling and the menu driven software. Kits for existing BBC micros are also available.

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● From the Macclesfield company HPLC Technology, the LC 2015 ternary gradient HPLC solvent pump and programmer enables the user to vary not only the isocratic mixtures, but to perform binary gradient elution with a 3-solvent mixture without the need for microprocessor-based control. Features include pulseless operation, flow of 0.1 ml per min, maximum pressure of 6,000 p.s.i. and the capacity for full external control.

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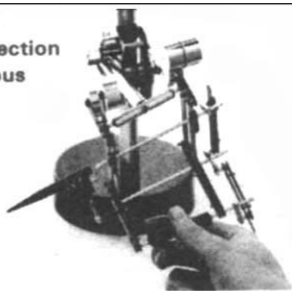
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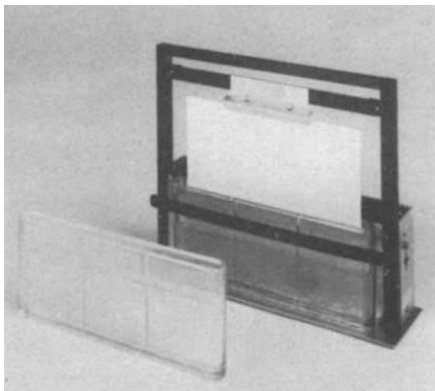
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The CAMAG scanner for TLC.

● The CAMAG automatic computer-controlled Scanner II + HP 9816 set up is a TLC evaluation tool featuring automatic scanning track location with optimization of each sample zone, automatic evaluation via peak height area, floppy disk storage of all raw data, dual wavelength scanning and video screen integration. For every assignment, the system gives a print out of a complete analysis report.

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● The Dionex range of ion chromatographs is soon to be augmented by a new advanced IC System, the AutoIon 300. This will be very user-friendly, enabling work methods to be quickly established and stored but have the facility to easily edit the parameters. The traditional role of IC to separate both anions and cations has been extended by Dionex to allow the analyst to measure amino acids in carbohydrates in even the most complex media such as fermentation broths.

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● A robot sampler for HPLC sample introduction has been announced by Varex Corporation. The ROSA robot sampler will automatically introduce samples from 0.2 microlitres to 2 millilitres into any liquid chromatograph, microbore or a conventional HPLC column. Samples may be injected either sequentially, or at random, with up to 10 repetitive injections from each vial.

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NOTES AND CATALOGUES

Circle the appropriate Reader Service Card numbers for free copies of the following applications notes and other literature.

● Now available on request from Bio-Rad is the latest issue of "Bio-Radiations". The 8-page issue contains details of a new Protein A-horseradish peroxidase conjugate immunoblot assay kit for the detection of nitrocellulose-bound immune complexes, and new affinity purified second antibody enzyme conjugates for ELISA and immunoblotting techniques. Application reports cover the purification of T-cell receptor components by high performance hydroxylapatite chromatography and optimizing protein separations using hydrophobic interaction HPLC. No. 130.

● A new 20-page issue of "Waters Chromatography Notes" featuring the latest instruments, columns and applications for high performance liquid chromatography is available from Waters Chromatography Division of Millipore Corporation. In addition to products, the latest issue contains new HPLC applications information for a variety of industries including biotechnology, electronics, food, beverage, grain, polymer, environmental, pharmaceutical and power. No. 131.

● Packard Instrument Ltd's two most recent "GC Application News Bulletins" each describe a particular analysis. The first describes the characterization of beeswax using the capillary liquid on-column injection technique and a deactivated fused silica retention gap with single ferrule column connection. The second describes the analysis of various trade preparations of pyrethroid insecticides using the capillary liquid on-column injection technique and an electron capture detector. No. 132.

● LDC/Milton Roy's new 24-page catalogue describes the complete line of LDC/Milton Roy HPLC products featuring individual instruments, components, options, replacement parts, columns and related accessories. No. 133.

● Three new application sheets from GOW-MAC Instrument Company cover solvent mixture analysis on capillary columns with micro TCD, nitrogen and phosphorous in a complex matrix at low level with TID, and flue gas analysis. The application sheets are part of a series, "Peak to Peak, Applied Separation Techniques"; each consists of a brief introduction, the equipment used including column data, operating conditions, and the analysis procedure. Readers requesting these sheets will also receive a full list of the other sheets available. No. 134.

● A 12-page brochure (Publication 514) gives details of Amicon's new line of silica packings for use in normal and reverse-phase liquid chromatography. In addition to detailed characteristics of the media, the publication offers a Selection Guide for desired applications, with emphasis on scale-up from laboratory to large, preparative applications. No. 135.

● LKB's complete line of chemicals and consumables for electrokinetic separations is listed in a 16-page catalogue. Products include prepared electrofocusing gels; Ampholine carrier ampholytes; gel media; molecular weight standards; stains and dyes. The catalogue also contains application information. No. 136.

● BDH and Baird and Tatlock have brought together their chromatography product ranges and included them in one 112-page catalogue. Many new products are included in the catalogue which details: HPLC instruments, columns, sorbents and reagents; TLC/HPTLC instruments, pre-coated plates, sorbents and reagents; preparative column chromatography. No. 137.

● Pharmacia Fine Chemicals has produced a large coloured poster entitled "Guide to Biological Purification and Isolation". The poster describes the principles and techniques, products used and the results to be expected for the isolation of a wide variety of biological substances, particles and cells. It describes the types of cells, subcellular particles and biomolecules that can be isolated, and the different methods of separation used. No. 138.

● J.T. Baker's 60-page catalogue "Products for Chromatography" lists hundreds of new products. Major topics covered include sample preparation, HPLC, thin layer chromatography and preparative column chromatography. No. 139.